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UNIVERSITY OF ALBERTA

SEX STEROIDS IN THE NEUROENDOCRINE REGULATION OF GONADOTROPIN AND GROWTH HORMONE SECRETION IN THE GOLDFISH

by

Vance L. Trudeau ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Zoology
Edmonton, Alberta
Spring 1992

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To whom it may concern,

Permission is hereby granted to Vance L Trudeau to use any scientific research which resulted from collaborative work involving the aforesaid person and myself in his doctoral thesis. This includes, but is not limited to, all work concerning dopamine and γ-aminobutyric acid turnover in response to steroids in goldfish, effects of domperidone on dopamine in goldfish pituitary and work involving taurine and gonadotropin release in goldfish.

Sincerely,

Duff Sloley

Brian Duff Sloley, Ph.D.

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December 13, 1991

TO WHOM IT MAY CONCERN

This is to confirm that Mr. Vance L. Trudeau has my full permission to include published collaborative research in his Ph.D. Thesis.

R. E. Peter,
Professor and Chairman

REP:rs

Anderson On-Lam Wong
Department of Zoology,
Biological Sciences Building,
University of Alberta.

24th October, 1991

Faculty of Graduate
Studies and Research,
University Hall,
University of Alberta.

Dear Sir,

This is to certify my approval for Mr. Vance Trudeau to include the data of in vitro interactions of dopamine D2 agonist LY171555 and steroids on gonadotropin-releasing hormone stimulated gonadotropin release in the goldfish as part of his Ph.D. thesis.

Yours sincerely,

And Wong

(Anderson Wong)

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Dear Sirs:

This is to permit Vance Trudeau to include in his Ph.D. Thesis data from the experiments looking at TRH and GnRH stimulation of GH release in vitro that we did in collaboration.

Sincerely yours,

Gustavo M. Somoza

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Biological Sciences Bldg.,
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October 24, 1991

Graduate Studies and Research
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Dear Sir/Madame,

Permission is hereby given to Mr. Vance Trudeau to use any scientific research which has been conducted collaboratively with Mr. Trudeau and myself towards his Ph.D thesis. This includes, but is not limited to, the studies on thyrotropin-releasing hormone and growth hormone release in the goldfish.

Yours sincerely,

Carol S. Nahorniak

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Dear Sir: October 14, 1991

This letter will confirm that we wish to provide the permission to Mr. Vance Trudeau to include our collaborative data and the paper titled " Testosterone potentiates the serum gonadotropin response to gonadotropin-releasing hormone in the common carp(Cyprinus carpio) and Chinese loach(Paramisgurnus dabryanus)" which has been accepted into Canadian Journal of Zoology recently as the appendix in his pH. D. thesis.

With best regards.

Yours sincerely

Lin Hao Ren

Lin Hao Ren
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October 18, 1991

The Faculty of Graduate Studies
University of Alberta
c/o Mr. Vance L. Trudeau
Department of Zoology
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Dear Sir/Madam:

Mr. Vance Trudeau has completed studies in collaboration with my laboratory on the effects of gonadal steroids on binding characteristics of gonadotropin-releasing hormone receptors in the goldfish pituitary. These studies were performed as part of Mr. Trudeau's studies leading to a Ph.D. degree at the University of Alberta.

Authorization is hereby given for inclusion of these results in Mr. Vance Trudeau's thesis to be submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Department of Zoology, University of Alberta.

Please do not hesitate to contact me if you require further information.

Yours sincerely,

Hamid R. Habibi, Ph.D.
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Celebrate!
25
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C.K.Murthy
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Edmonton
Oct 29, 1991.

To

The Faculty of Graduate Studies and Research
University of Alberta,
Edmonton

Dear Sir,

I hereby grant my approval for Mr. Vance Trudeau to include the data pertaining to studies on gonadotropin-releasing hormone receptors which we did together, as a part of his Ph.D thesis.

Yours sincerely,

(C.K.Murthy)

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DEGREE: Doctor of Philosophy

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Vance Trudeau
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Date: December 18, 1991

UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of graduate Studies and Research for acceptance, a thesis entitled: **SEX STEROIDS IN THE NEUROENDOCRINE REGULATION OF GONADOTROPIN AND GROWTH HORMONE SECRETION IN THE GOLDFISH** submitted by Vance L. Trudeau in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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(Dr. R.E. Peter, Supervisor)

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Date: December 4, 1991

ABSTRACT

The effects of testosterone (T) and estradiol (E_2) on serum gonadotropin (GTH) and growth hormone (GH) concentrations were investigated throughout the seasonal reproductive cycle of the goldfish. Gonadal size, pituitary GTH content, basal and gonadotropin-releasing hormone (GnRH)-stimulated serum GTH, and basal serum GH levels were all maximal in spring, and minimal in summer-early autumn. Intraperitoneal implantation of T and E_2 produced serum steroid levels similar to those for during natural ovulation in goldfish. Both T and E_2 potentiated the pituitary GTH release-response to GnRH without affecting basal serum GTH levels. Testosterone potentiated the serum GTH response to GnRH throughout the reproductive cycle, whereas E_2 was active in sexually regressed females only. The potentiating effect of T was dependent on aromatization to E_2 since T positive action was blocked by an aromatase inhibitor. The positive effects of T on GnRH-induced GTH secretion *in vitro* is protein synthesis dependent and appears to be independent of changes in pituitary content of immunoreactive GTH, brain and pituitary content of GnRH and pituitary GnRH receptor affinity or binding capacity.

Estradiol but not T stimulates increases in serum growth hormone levels throughout the reproductive cycle. GnRH stimulates GTH and GH secretion from pituitary fragments *in vitro* whereas thyrotropin releasing hormone (TRH) stimulates only GH release. E_2 treatment potentiates the *in vitro* GH release-responsse to both TRH and GnRH.

Dopamine inhibits and norepinephrine stimulates GnRH and GTH release in the goldfish by actions in the brain and pituitary. Sex steroids interact with catecholaminergic systems in the goldfish by altering turnover rates in dopaminergic and norepinephrinergic neurons in the telencephalon-preoptic area, hypothalamus and pituitary which may affect GnRH neuron function and GTH release.

The goldfish brain and pitutary contain the amino acid neurotransmitters γ-aminobutyric acid (GABA), glutamate (GLU) and taurine (TAU), and each of these substances stimulates GTH release. Sex steroids modulate GABA action by affecting pituitary GTH release in response to GABA and by regulating GABA synthesis rates in the brain and pituitary. Steroids potentiate TAU-stimulated GTH release but the mechanisms underlying this action are unknown. Glutamate may act through N-methyl-D-asparate receptors to stimulate GTH release, an action not affected by sex steroids.

These results demonstrate that sex steroids interact at multiple sites within the brain-pituitary axis to control GTH and GH secretion in goldfish. A model describing the involvement of sex steroid feedback in the neuroendocrine regulation of reproduction and growth in teleosts is proposed.

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There is one exceptional person whose contributions to the success of my thesis are not seen on any of the pages included within. Her continuous support, encouragement and love throughout the writing and submission of my thesis has made it all worthwhile. Thank you, Eve.

Finally, I would like to express my gratitude to the Alberta Heritage Foundation for their generous financial support during my study period at the University of Alberta.

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LIST OF COMMON ABBREVIATIONS

Common abbreviation	Definition
ATD	1,4,6-androstatrien-3,17-dione
CA	catecholamine
CE	catecholestrogen
COMT	catechol-O-methyl-transferace
DA	dopamine
DA-TOR	dopamine-turnover rate
DHT	dihydrotestosterone
DOM	domperidone
E_2	estradiol-17β
ED_{50}	effective dose (50 % of maximal)
GABA	γ-aminobutyric acid
GRF	growth hormone releasing factor
GH	growth hormone
GLU	glutamate
GnRH	gonadotropin-releasing hormone
cGnRH-I	chicken gonadotropin-releasing hormone-I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2)
cGnRH-II	chicken gonadotropin-releasing hormone-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2)
l-GnRH	lamprey gonadotropin-releasing hormone (pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH_2)
mGnRH	mammalian gonadotropin-releasing hormone (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)
sGnRH	salmon gonadotropin-releasing hormone (pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2)
sGnRH-A	salmon gonadotropin-releasing hormone agonist (pGlu-His-Trp-Ser-Tyr-*D*-Arg-Trp-Leu-Pro-N-ethylamide)
GSI	gonadosomatic index
GTH	gonadotropin-II
GVG	γ-vinyl-γ-aminobutyric acid
hCG	human chorionic gonadotropin
HPLC	high performance liquid chromatography
HYP	hypothalamus

i.c.	intracerebrovetricular injection
i.p.	intraperitoneal injection
11-KT	11-keto-testosterone
LH	luteinizing hormone
LHRH-A	luteinizing hormone-releasing hormone agonist (pGlu-His-Trp-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-ethylamide)
NIL	neurointermediate lobe of pituitary
NMA	N-methyl-D,L-aspartic acid
NPY	neuropeptide Y
MAO	monoamine oxidase
NE	norepinephrine
NE-TOR	norepinephrine-turnover rate
P_4	progesterone
PD	pars distalis of the pituitary
PIT	pituitary
RIA	radioimmunoassay
SRIF	somatostatin (somatotropin-release inhibiting factor)
T	testosterone
TOR	turnover rate
TRH	thyrotropin-releasing hormone

1. GENERAL INTRODUCTION

A brief history of sex steroid feedback

In 1932, Moore and Price demonstrated the existence of a reciprocal relationship between the testes and ovary and the functioning of the anterior pituitary gland of the rat. From extensive experimentation they succinctly concluded that:

> "Gonad hormones, of either sex, exert a depressing effect upon the hypophysis which results in a diminished amount of the sex-stimulating factor available to the organism"

Their observations served as the initial demonstration of sex steroid negative feedback. In contrast Holweg (1934) proposed that estrogen could exert positive actions on gonadotropic function. Holweg demonstrated that estrogen administered to prepubertal rats causes premature luteinization of the ovary, an effect shown to be dependent on the presence of a pituitary gland (Westerman and Jacobsohn, 1938). How can sex steroids exert both positive and negative feedback? Zondek (1935) was likely the first to explain the apparent paradox of sex steroid positive and negative feedback, suggesting that estrogenic effects may be dose dependent. Davidson (1969; 1983) presents a clear summary of the early developments in the debate of stimulatory versus inhibitory feedback mechanisms.

Although Geoffrey Harris is best known for his establishment of the concept of neural control of pituitary function (Harris, 1955), he also raised the possibility that sex steroid negative feedback could be at the hypothalamic as well as the pituitary level (Harris, 1964; 1972). Flerko and Szentagothai (1957) demonstrated that implantation of minute amounts of ovarian tissues in the hypothalamus and not the pituitary reduced uterine weight (index of steroid secretion and negative feedback) and thus concluded that sex steroids act centrally to control gonadotropin secretion. In contrast, Bogdanove (1963;1964) placed similar ovarian grafts or depots of estrogen in the pituitary of ovariectomized rats and concluded that sex steroids act directly on the pituitary to reduce gonadotropin secretion.

Despite the extemely sophisticated methods now available in endocrinology, neurophysiology and molecular biology, the debate on the sites and mechanisms of sex steroid feedback have only intenstified. The extensive literature on positive and negative feedback in the rat has recently been reviewed by Fink (1988) and Kalra and Kalra (1983; 1989). Others have reviewed the literature on the effects of sex steroids in control of gonadotropin secretion in other animal models such as the sheep (Karsh, 1987), domestic chicken (Sharp, 1983) and primate (Knobil and Hotchkiss, 1988; Plant, 1988). In marked contrast, there is very little information on the role of sex steroids in the neuroendocrine regulation of gonadotropin secretion in the largest of vertebrate groups, the fishes. Kopéc (1918) was likely the first to gonadectomize fish and demonstrated that nuptial coloration in *Phoxinus* is lost following removal of the testes or ovaries. Van Oordt (1923) followed with similar observations in the 10-spined stickleback. For decades, the relationships between brain and/or pituitary function and gonadal function in fish was confined to correlative observations during reproductive/seasonal cycles. It was not until the late 1960's that sex steroids were isolated from fish sources (see Ozon, 1972a,b for review) and fish biologists entered the debate on steroid feedback (see Egami and Arai, 1965; McBride and van Overbeeke, Nagahama and Yamamoto, 1969 and 1969; van Overbeeke and McBride, 1971). Peter (1970) introduced the concept of hypothalamic control of teleost pituitary-gonadal function. Development of radioimmunoassays for steroids and isolation of gonadotropin in fish quickly added to the endocrine data on pituitary-gonadal correlations in fish. In the following sections neuroendocrine regulation of gonadotropin (GTH) secretion in teleosts is reviewed. In addition, temporal variations in pituitary-gonadal function and existing evidence for positive and negative effects of sex steroids on GTH secretion in teleosts are also reviewed.

Neuroendocrine regulation of GTH secretion in teleost fish

The neuroendocrine regulation of GTH secretion in teleost fish has been reviewed extensively (Peter, 1983; Peter et al., 1986; Peter et al., 1990). In goldfish, gonadotropin-releasing hormone (GnRH), neuropeptide Y, norepinephrine and serotonin stimulate GTH secretion, whereas dopamine is a potent inhibitor of GTH secretion.

Actions of GnRH

It is now well established that GnRH and its analogues stimulate GTH release from the teleost pituitary *in vivo* and *in vitro* (Peter, 1983; Peter et al., 1987). Gonadotropin-releasing hormone acts through a specific pituitary receptor to elicit GTH secretion. Both high affinity/low capacity and low affinity/high capacity binding sites have been characterized in goldfish pituitary (Habibi et al., 1987) and it is the high affinity GnRH binding sites which are involved in GTH release (Habibi et al., 1989a). In the African catfish (*Clarias gariepinus*) only a high affinity GnRH receptor has been identified (De Leeuw et al., 1988). Salmon GnRH (sGnRH), mammalian GnRH (mGnRH), chicken GnRH-I (cGnRH-I) , cGnRH-II and lamprey GnRH (l-GnRH) at one high dosage have similar GTH-releasing activities *in vivo* in goldfish treated with dopamine antagonists

(Peter et al., 1985; Peter et al., 1987). In contrast, in experiments using *in vitro* perifusion of pituitary fragments, important differences in GTH-releasing activity and receptor binding affinity of various GnRH molecules have been shown (Habibi et al., 1989a). This may be due to a more refined ability to quantify subtle differences in GTH-release using a perifusion system or may relate to the presence and absence of a serum GnRH binding protein (Huang and Peter, 1988) *in vivo* and *in vitro*, respectively. Several synthetic analogues have been tested for GTH-releasing activity; amino acid substitution at position 8 in mGnRH with strongly negative amino acids (i.e. Leu, His, Trp) decreases activity, but substitution with a neutral amino acid (i.e. Asp, Met, Phe) does not (Peter et al., 1987). GTH-releasing activity of GnRH and its analogues has also been shown in trout (Crim and Evans, 1979), walleye (*Stizostedion vitreum*; Pankhurst et al., 1985), African catfish (*Clarias gariepinus*; de Leeuw et al., 1987) and sea bream (*Sparus aurata*; Zohar et al., 1989).

GnRH action on GTH release *in vivo* is prolonged in teleosts compared to mammals. In goldfish, levels of GTH may be elevated for up to 24-48 hours following intraperitoneal injection of certain agonists (Peter, 1983; Peter et al., 1985; 1987). Differences in releasing activity of the various forms of GnRH may relate to differences in pituitary GnRH receptor binding affinities (Peter et al., 1987; Habibi et al., 1989a) and/or resistance to metabolic degradation (Zohar et al., 1989; 1990). However, the binding to the high affinity GnRH binding site by sGnRH and the superactive analogue, [D-Ala^6-Pro^9-Net]-mGnRH are similar, so the difference in their activity may be due to differential degradation (Habibi et al., 1989a). More recently, a GnRH binding protein has been characterized in goldfish serum (Huang and Peter, 1988) and may confer resistance to degradation of GnRH and account, at least partially, for its prolonged effects on GTH secretion *in vivo*.

The response to injected GnRH varies seasonally in teleost fishes. Sexually mature (pre-spawning) rainbow trout are more responsive to GnRH than fish at other stages of the sexual cycle (Weil et al., 1978). In carp, the greatest response to GnRH was during the spring spawning season and minimum response was in winter when the fish were sexually inactive (Weil et al., 1980). In addition, goldfish are more responsive to GnRH in the spring spawning season than at other times of the year (Sokolowska et al., 1985a; 1985b). Increasing water temperature from 12^o C to 20^o C also potentiates GnRH-induced GTH release (Sokolowska et al., 1985a; 1985b). Higher pituitary contents of both high and low affinity sites were found during the later stages of gonadal recrudescence (Habibi et al., 1989b), at a time when the levels of endogenous steroids are increasing in the goldfish (Kagawa et al., 1983). Seasonal variations in GnRH receptor capacity of the high affinity sites were highly correlated with variations in GnRH-induced GTH release *in vivo* (Habibi et al., 1989b). The mechanism involved in the seasonal increase in GnRH responsiveness and GnRH receptor capacity in goldfish is not known. This may be related to seasonal changes in sex steroid levels since in the rat, sex steroid feedback on gonadotropin secretion may be mediated by changes in pituitary responsiveness to GnRH (Negro-Vilar et al., 1973; Drouin et al., 1976; Drouin and Labrie, 1981; Kalra and Kalra, 1983) and/or GnRH receptor numbers (Clayton and Catt, 1981; Clayton et al., 1985; Conn et al., 1987).

Localization of GnRH

Immunocytological evidence from several laboratories strongly implicates the anteroventral preoptic region of the brain as the primary location of GnRH perikarya in teleosts (Peter, 1983; Kah et al., 1986; Peter et al., 1986). The presence of immunoreactive perikarya in the olfactory tracts, ventral telencephalon, ventro-lateral hypothalamus and rostral midbrain tegmentum have also been demonstrated in the goldfish brain (Kah et al., 1986). In addition, major projections in the preoptico-hypophyseal and periventricular pathways have been discovered; GnRH neurons directly innervate GTH cells in the pars distalis (Kah et al., 1986).

Two molecular forms of GnRH have been identified in the extracts of various brain areas, spinal cord and pituitary of the goldfish. The chromatographic and immunologic properties of these GnRH molecules resembles those of chicken GnRH-II (cGnRH-II) and salmon GnRH (sGnRH). The proportion of the cGnRH-II-like molecule is higher in caudal brain areas (Yu et al., 1988).

Involvement of biogenic amines in GTH secretion

Aminergic fibers have been shown to innervate gonadotrophs of many teleosts, including the goldfish (Kaul and Vollrath, 1974) and it is known that norepinephrine and serotonin stimulate whereas DA inhibits GTH secretion in this species (Peter et al., 1986; Peter et al., 1990).

Norepinephrine (NE)

Intraperitoneal or intraventricular injection of NE elevates serum GTH levels in female goldfish in sexually regressed or early gonadal recrudescence stages, but not at other times of the seasonal sexual cycle (Chang and Peter, 1984); this differential responsiveness may be due to seasonal changes in circulating steroid levels. More recently, Yu et al. (1991b; Yu and Peter, 1991) have demonstrated that NE can stimulate GnRH release from brain slices *in vitro* and this may be a possible mechanism for the central stimulatory effect of NE on GTH release *in vivo*. In addition, Chang et al. (1991) have shown that NE can stimulate GTH release directly from dispersed anterior pituitary cells in culture.

Dopamine (DA)

The involvement of DA in the neuroendocrine regulation of GTH in goldfish was first demonstrated by Chang et al. (1983a,b,c). Injection of DA synthesis inhibitors and DA antagonists such as pimozide (PIM), metocloprimide, and domperidone (DOM), increase serum GTH levels whereas injections of DA or the DA agonist, apomorphine, decrease GTH levels *in vivo* in goldfish (Peter et al., 1986; Omeljaniuk et al., 1987). In addition, goldfish pituitary fragments respond to DA by decreasing GTH release *in vitro* (Chang and Peter, 1984). Not only does DA inhibit basal GTH secretion in goldfish, but also GnRH-induced release of GTH both *in vivo* and *in vitro* (Peter et al., 1986). Dopamine binding sites in goldfish pituitary have been characterized and are similar to the mammalian type 2 DA receptors (Omeljaniuk and Peter, 1989). These observations, together with the fact that pituitary gonadotrophs are directly innervated by DA-immunoreactive fibres (Kah et al., 1984), indicate that DA acts directly at the pituitary level to inhibit GTH secretion (Peter et al., 1986). The effects of DA to inhibit GTH release also involves actions on GnRH neurons since DA inhibits GnRH release from preoptic-anterior hypothalmus and pituitary slices *in vitro* (Yu et al., 1991b).

In contrast to the effects of DA and its agonists, antagonists of DA greatly potentiate GnRH-induced GTH release *in vivo* (Peter et al., 1986). Furthermore, the magnitude of the response to GnRH in combination with a DA antagonist (PIM) increases with gonadal development, maximal responses being found in fish in the late stages of gonadal recrudescence (Sokolowska et al., 1985b). The authors suggested that the inhibitory influence of DA increased with seasonal sexual development in goldfish and this may involve seasonal changes in sex steroid levels.

The GTH surges induced by comcommittant GnRH and DA antagonist treatment also induce ovulation in goldfish (Sokolowska et al., 1984), common carp (Billard et al., 1983), loach (*Paramisgurnus dabryanus*; Lin et al., 1985) and African catfish (Richter et al., 1987). This suggests that the GTH surge during natural ovulation may be due, at least partly, to a concommitant reduction in dopaminergic inhibition and increased GnRH release prior to the ovulatory GTH surge (Yu et al., 1991a). This may involve gonadal steroids since E_2 and T secretion increases immediately prior to ovulation in goldfish (Kobayashi et al., 1987).

Serotonin (5HT)

Although little is known about the role of 5HT in control of GTH release in teleosts, 5HT-immunoreactive fibres have been demonstrated in the pars distalis of the goldfish (Kah and Chambolle, 1983) and 5HT stimulates GTH release *in vivo* (Somoza et al., 1988) and *in vitro* (Somoza and Peter, 1991) in the goldfish. It has been shown that 5HT stimulates GnRH release from nerve terminals in the pituitary, and from GnRH neurons in the preoptic-anterior hypothalamic region of goldfish (Yu et al., 1991b).

Temporal variations in serum GTH and sex steroid levels in teleost fish

Annual cycles

Seasonal increases in gonadal size of precocious male Atlantic salmon parr (*Salmo salar*) are correlated with increases in both pituitary and plasma GTH levels during the autumn (Crim and Evans, 1978). In domesticated male rainbow trout (*Oncorhynchus mykiss=Salmo gairdneri*), seasonal increases in testosterone (T) coincide with periods of spermiation in October, and elevations of 11-ketotestosterone (11-KT) occur at the completion of spermatogenesis in February (Scott et al., 1980). Since T may be an intermediate product in the synthesis of 11-KT (Ozon, 1972a), seasonal increases in T followed by 11-KT elevations are expected. Similar patterns of T and 11-KT secretion (Kime and Manning, 1982) have been observed for male brown trout (*Salmo trutta*). Plasma 17α, 20ß-dihydroxy-4-pregnen-3-one (17, 20βP) levels increase during the beginning of the spawning season (October), remain high during spermiation and decrease by mid-November in amago salmon (*Oncorhynchus rhodurus*). These changes in 17, 20βP were associated with seasonal changes in gonadal size (Ueda et al., 1983). In post-ovulatory female Atlantic salmon decreases in both T and estradiol-17ß (E_2) are accompanied by increases in plasma GTH (Stuart-Kregor et al., 1981). This is also the case in female rainbow trout during later stages of ovarian development, suggesting that the release of GTH during ovulation is a result of decreased negative feedback by E_2 (Bromage et al., 1982)

In female goldfish, levels of T and E_2 increase in March and are relatively high until April, and decline in the post-ovulatory period (Kagawa et al., 1983). The progestogens, 17α-hydroxy-progesterone (17P) and 17, 20βP increase just prior to ovulation and decrease 1 day following ovulation (Kagawa et al., 1983). Concurrent changes in GTH, gonadal size and gonadal steroids throughout the seasonal reproductive cycle have been documented for goldfish (Kobayashi et al., 1986a). For female goldfish in Japan, peak values for gonadal size are usually found in May and the spawning period is from May to June. Plasma GTH levels are low during gonadal recrudescence and increase during the spawning period. Plasma E_2 levels peak in April and T levels are maximal in May. Kobayashi et al. (1986a) suggest the delay in T secretion was due to an increased number of oocytes in the tertiary yolk globule stage that produce T but not E_2. After spawning both T and E_2 levels decline. In male goldfish (Kobayashi et al. 1986a), gonadal size increases gradually from October through May and decreases after spawning. Plasma GTH levels in males, as in females, increase in May when levels of 11-KT and T are maximal.

Spawning cycles

The endocrine events associated with spawning have been characterized for carp (*Cyprinus carpio*; Santos et al., 1986), white suckers (*Catostomus commersoni;* Scott et al., 1984; Stacey et al., 1984), goldfish (*Carassius auratus*; Stacey et al., 1979; Kagawa et al., 1983; Kobayashi et al., 1986b; Kobayashi et al., 1987), Atlantic salmon (*Salmo salar*; Stuart-Kregor et al., 1981) and rainbow trout (*Oncorhyncus mykis*; Fostier et al., 1978). A common finding in these studies is that blood levels of GTH increase at some time prior to ovulation. In female common carp, (Santos et al., 1986), GTH increases in the afternoon approximately 10 hours prior to ovulation and peak levels are found at the time of ovulation. Blood concentrations of 17,20βP rapidly increase with the commencement of the preovulatory GTH surge, suggesting that GTH stimulates steroidogenesis at this time. Only small changes in E_2 occur in the periovulatory period of common carp; E_2 increases slightly during the GTH surge. Lin et al. (1986) noted that in ovulatory common carp, GTH levels increase progressively throughout the night and reach maximal levels at the end of the dark period. In female goldfish, the ovulatory period is characterized by gradual increases in GTH late in the day which are followed by a GTH surge and ovulation during the night (Stacey et al., 1979; Kobayashi et al., 1987). After ovulation in goldfish, GTH levels drop rapidly and are low the following day. Plasma 17,20βP and T peak before ovulation and decrease by the time of ovulation. Plasma E_2 levels increase gradually prior to ovulation, are maximal during the GTH surge and returns to basal levels on the day following ovulation. In male goldfish, plasma levels of T and 17, 20βP increase concurrent with the GTH whereas 11-KT remains low during spawning activity (Kobayashi et al., 1986b). Increased GTH stimulates milt production (Kyle et al., 1985) and this effect is mediated by the testicular steroids T and 17,20βP (Kobayashi et al., 1986a). In male goldfish GTH increases in synchrony with that of females during spawning (Stacey et al., 1989). The co-ordination of gonadal and behavioural events leading to spawning in goldfish is mediated via pheromonal mechanisms (Kobayashi et al., 1986c; Sorensen and Stacey, 1991).

In female rainbow trout, plasma E_2 levels decrease prior to and during the periovulatory period (Fostier et al., 1978; Scott et al., 1983) whereas T, 17,20βP and 17P increase in concert with GTH (Fostier et al., 1981; Scott et al., 1983). Several researchers suggest that the decrease in E_2 levels triggers the GTH surge and ovulation (Bromage et al., 1982; Fostier et al., 1983; Scott et al., 1983) by decreased negative feedback on the pituitary gonadotroph; however, experimental evidence for this is lacking. Artificially elevated E_2 levels do not influence GTH or ovulation in the goldfish (Pankhurst and Stacey, 1985; Kobayashi et al., 1987) although E_2 increases at the time of the GTH surge in goldfish (Kobayashi et al., 1987) and the white sucker (Scott et al., 1984). Stacey et al.

(1984) point out that fish in the final stages of oocyte maturation exhibit increased GTH levels with no apparent decrease in E_2. They suggested that this argues against a negative feedback effect of E_2 at the time of ovulation.

The significance of changes in gonadal steroid levels in relation to pituitary gonadotroph function is not known. In contrast, the involvement of T and 17, 20βP in spermiation, 11-KT in male secondary sexual characteristics, 17, 20βP in final oocyte maturation and E_2 in vitellogenesis, are well documented for teleosts, and have been reviewed previously (Fostier et al., 1983; Ng and Idler, 1983; Nagahama, 1989).

Influence of gonadal steroids in the control of GTH secretion in teleost fish

Evidence for negative effects of gonadal steroids on GTH secretion

The classical approach to the study of steroid feedback mechanisms and the control of GTH secretion involves castration, followed by steroid replacement therapy and subsequent assessment of alterations in pituitary histology and plasma GTH levels. Castration of sockeye salmon (*Oncorynchus nerka*) results in degranulation of periodic acid-schiff (PAS)-positive cells, the putative gonadotrophs (van Overbeeke and McBride, 1971). Treatment of castrated salmon with either 11-KT, 17-methyltestosterone, E_2 or E_2 cypionate induced reappearance of cytoplasmic granules in the gonadotrophs. Various steroid treatments decrease PAS-staining granule activity in the pituitary of the guppy (*Poecilia reticulata*; Sage and Bromage, 1970). Similarly, long-term immersion treatment (28 days) with methyltestosterone decreases gonadotroph activity (van den Hurk and Testerink, 1975) of male black mollies (*Mollienisia latipinna*). Although these studies suggest inhibitory effects of gonadal steroids, it is not entirely clear since pituitary or serum GTH levels were not measured.

Castration of mature male rainbow trout results in increases in plasma GTH levels during the spawning season and smaller increases at other times of the year (Billard et al., 1977; Billard et al., 1978; Billard, 1978). Intraperitoneal implantation of T or E_2 in castrated trout reduces GTH levels. It was suggested that androgens must be aromatized before being effective in suppression of GTH. However, non-aromatizable androgens may also be involved since high plasma GTH levels can be reduced in castrate trout by pituitary implantation of 11-KT, a non-aromatizable androgen (Billard, 1978). Removal of the ovaries in trout near the end of vitellogenesis causes an increase of plasma GTH that cannot be suppressed by E_2 administration (Bommelaer et al., 1981). In those fish undergoing germinal vesicle migration, ovariectomy resulted in elevated GTH in approximately 50% of fish and this increase was E_2-suppressible. As evidenced by Bommelaer et al. (1981), there may be seasonal variation in the negative feedback sensitivity of GTH secretion to E_2 in salmonids. However, it seems that the GTH responses following gonadectomy in trout species are variable, and the risk of incomplete gonad removal (Billard et al., 1982) must be acknowledged.

More recent data in the African catfish (*Clarias gariepinus*) have demonstrated clearly that sex steroids inhibit GTH release in this species. Castration of adult male catfish leads to increased plasma GTH levels, decreased pituitary GTH content and degranulation of the gonadotrophs (De Leeuw et al., 1987). Treatment of castrated fish with aromatizable androgens (T or androstenedione) in silastic capsules restored pituitary function to pre-castration levels. Non-aromatizable steroids (dihydrotestosterone; DHT or 11ß-hydroxy-androstenedione) did not abolish the castration response.

De Leeuw et al. (1987) have suggested that the negative feedback actions of estrogen on GTH secretion are mediated by its effects on dopamine (DA). Under De Leeuw's hypothesis, estrogens are metabolized to the catecholestrogens (CE) and compete for catecholamine-O-methyl-transferase (COMT), therefore decreasing DA catabolism and subsequently reducing GTH secretion via increased DA inhibition. COMT is widely distributed in the brain and pituitary of the African catfish (Timmers and Lambert, 1989), but its significance to DA catabolism in fish has not been established. Furthermore, the direct effects of CE on GTH release have not been examined in any teleost species.

Other lines of evidence for steroid negative feedback on GTH secretion involve the use of various putative anti-estrogen compounds (i.e clomiphene citrate and Tamoxifen). Clomiphene citrate induces ovulation in goldfish (Pandey and Stacey, 1975) and implantation of clomiphene citrate or Tamoxifen (ICI 46474) into the nucleus lateralis tuberis of the hypothalamus and pituitary of goldfish results in GTH release (Billard and Peter, 1977). However, the assumption that clomiphene citrate and Tamoxifen are anti-estrogenic may not be true in teleosts; in none of these studies were fish treated with estrogen and anti-estrogen simultaneously. In addition, hypothalamic and pituitary implantation may have damaged the pituitary stalk and disrupted inhibitory dopaminergic inputs into the pituitary (Peter et al., 1986) and caused GTH release.

Evidence for positive effects of gonadal steroids on GTH secretion

Evidence for the positive effects of gonadal steroids on GTH secretion comes primarily from studies on immature salmonids and immature eels. Implantation of T in the nucleus lateralis tuberalis of the hypothalamus and pituitary of the immature Atlantic salmon parr caused an increase in pituitary GTH concentrations (Crim and Peter, 1978); however, implantation of T in the nucleus preopticus had no effects on pituitary GTH concentrations. Pituitary GTH stimulation by T is greater in males than in females. These initial observations led to the hypothesis that T-stimulated GTH accumulation is involved in the onset of precocious sexual maturation in male Atlanic salmon. Intraperitoneal administration of T in male and female rainbow trout also stimulates pituitary GTH accumulation (Crim and Evans, 1979). In addition, *in vivo* treatment with T enhances GnRH-induced GTH secretion *in vitro* (Crim and Evans, 1980). Low pituitary GTH levels, characteristic of juvenile trout are elevated 7 days following perivisceral implantation of T, androstenedione, estrone, E_2 and estriol but not DHT, 11-KT, 11-ß-hydroxy-testosterone (11ß-OHT), 17P or 17,20β P (Crim et al., 1981). The implantation of an aromatase inhibitor reduced the pituitary GTH response to T. On this basis, Crim et al. (1981) concluded that androgens must be aromatized to exert their effects on GTH. The positive effects of endogenous gonadal steroids on pituitary GTH content was demonstrated by Crim et al. (1982); injection of GTH caused subsequent stimulation of plasma androgen levels and eventual accumulation of GTH in the pituitary of trout. In all of these studies, no changes in plasma GTH were noted. In subsequent experiments (Crim and Evans, 1983), however, it was shown that implantation of juvenile rainbow trout with T-containing silastic capsules for two months results in GTH release to the circulation. GnRH implants have no effects on plasma GTH levels, but when co-implanted with T, increased plasma GTH levels were observed after 1 month, suggesting a need for steroid priming of the pituitary for GTH release in immature fish (Crim and Evans, 1983). The mechanisms and sites of action of steroids may be at both pituitary and brain levels. In immature trout, T induces accumulation of GTH in pituitaries transplanted to the caudal musculature (Gielen and Goos, 1983) and increases GnRH-bioactivity in telencephalon and diencephalon extracts (Goos et al., 1986).

Positive effect of E_2 on morphological development of GTH cells has been demonstrated in the pituitary of immature female (Olivereau and Olivereau, 1979a) and male European freshwater eels (*Anguilla anguilla*; Olivereau and Olivereau, 1979b). Estradiol treatment also stimulates prolactin and growth hormone cells. These treatments, however, involve injection of 150 µg E_2 every 2 days for 80 days, and may not represent the physiological situation. In other studies on the female European eel, however, lower doses of E_2 for shorter periods of time also increase pituitary GTH levels (Dufour et al., 1983). Estradiol increases radioimmunoassayable GnRH in the brain of the immature European eel (Dufour et al., 1986) and could be the mechanism whereby sex steroids regulate GTH production and secretion (Shin and Howitt, 1976; Kalra and Kalra, 1983; Kalra and Kalra, 1989; Melrose et al., 1987; Marshall et al., 1990) .

Introduction to the goldfish model

It was at this point in the chronology of sex steroid feedback in teleosts that I began the work outlined in the following chapters. The goldfish represents a good model to study the involvement of sex steroids in the control of pituitary function in teleosts since a great deal of basic information exists for seasonal reproductive cycles and neuroendocrine control of GTH release in this species. Owing to its small size, however, frequent blood sampling is not possible, making study of pulsatile GTH release difficult. This represents a major limitation, since an important mechanism of steroid action in other vertebrates is regulation of pulsatile release of gonadotropic hormones (Gallo, 1981; Karsh, 1987; Levine and Ramirez, 1980).

Classically, the initial studies of gonadal feedback should start with development of a gonadectomy/steroid replacement paradigm. However, although gonadectomy is possible in teleosts, the probability for incomplete removal and subsequent regeneration of the gonad is great. There is also considerable stress asssociated with the surgery, and the long recovery period necessary in goldfish precludes the study of short-term effects of gonadectomy. More important, however, is the validity of the gonadectomized model for the study of sex steroid feedback. In all species, the gonad is omnipresent, and long or short-term removal of this organ may not represent a physiological state. In gonadectomized mammals, gonadotropin secretion becomes less sensitive to control by GnRH and sex steroids compared to that in intact animals (Kalra and Kalra, 1989). Also, in castrates, gonadotropin secretion becomes independent of some neurotransmitter control mechanisms (Herdon et al., 1984) suggesting a pathophysiological rather than a physiological status of the long-term gonadectomized animal. It is now known that gonadectomy not only removes the influence of gonadal steroids but also non-steroidal factors that are important to regulation of pituitary function (deJong, 1988).

My initial studies (Trudeau et al., 1988) in the goldfish suggested that sex steroids do not affect basal GTH secretion. This was in marked contrast to the data of Bommelaer et al. (1981) and DeLeeuw et al. (1987) demonstrating that castration increases and subsequent steroid replacement decreases basal GTH secretion in fish. Outlined in Chapter 2 are the data on development of the gonad-intact, steroid-treated goldfish paradigm for the study of sex steroid feedback in the goldfish. The data demonstrate for the first time in an adult gonad-intact teleost that T, via aromatization to E_2, increases pituitary responsiveness to the neuropeptide GnRH without affecting basal GTH secretion. Data presented in Chapter 3 demonstrate that the positive effect of sex steroids involves changes in protein synthesis in the pituitary gland, and does not seem to be related to changes in GnRH levels in the brain or pituitary, or changes in pituitary GnRH receptor binding capacity or affininty. Since the catecholamines are important regulators of hypothalamo-hypophyseal function in mammals (Barraclough et al., 1984; Ramirez et al., 1984) and fish (Peter et al., 1986), Chapter 4 examines the influence of sex steroids on catecholamine turnover in the brain and pituitary in relation to GTH secretion. Recently, there has been a surge in interest in the role of amino acid neurotransmitters in the neuroendocrine regulation of pituitary function in mammals (McCann and Rettori, 1988; Plant, 1988). Data presented in Chapter 5 indicate that the amino acid neurotransmitters, γ-amino butyric acid and taurine, both present in the goldfish brain and pituitary, have a stimulatory role in the control of GTH secretion in goldfish. Furthermore, their actions are dependent on sex steroids. Finally, because there is considerable data indicating either a positive or reciprocal relationship between the reproductive and somatic axes in vertebrates (Hervey and Hervey, 1981; Sherry, 1981; Marchant and Peter, 1986) and sex steroids affect growth hormone (GH) secretion in the rat (Jansson et al., 1985), studies were initiated to examine the relationship between sex steroids and GH secretion in goldfish. Data presented in Chapter 6 demonstrate that E_2 stimulates, but T has no effects, on basal GH secretion in the goldfish. Together, the information presented herein lays the foundation for future studies on the neuroendocrine integration of the brain-gonadal axis in relation to reproduction and growth in cyprinid fish.

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2. TESTOSTERONE AND ESTRADIOL POTENTIATE THE SERUM GONADOTROPIN RESPONSE TO GONADOTROPIN-RELEASING HORMONE IN GOLDFISH*

Introduction

Gonadal steroids are important feedback regulators of gonadotropin (GTH) secretion and have been extensively studied in mammals (Karsh, 1987; Fink, 1988) and birds (Sharp, 1983). The gonadal steroids exert their effects by interacting with specific steroid binding sites in the brain and pituitary (McEwen et al., 1984). In the case of teleost fish very little is known about steroid feedback mechanisms, but steroid binding sites have been localized by autoradiographic methods in the brains of a variety of species. In platyfish and goldfish (Kim et al., 1978) labelled testosterone (T) and estradiol (E_2) have a similar distribution in the preoptic area, hypothalamus and in the pituitary. These areas are known to be involved in the control of GTH secretion in teleost fish (Peter, 1983).

Many of the actions of androgenic steroids (i.e. T) are mediated by their localized conversion within the vertebrate brain by reductase and aromatase enzymes to 5α-dihydroxy-testosterone (DHT) and E_2, respectively (Callard, 1983). The distribution of brain 5α-reductase and aromatase activities have been characterized in goldfish (Pasmanik and Callard, 1985) . The areas demonstrating high activity are the preoptic area, anterior hypothalamus, telencephalon and pituitary. The distribution of brain gonadotropin-releasing hormone (GnRH; Peter, 1983; Kah, 1986), steroid concentrating areas and aromatase activity exhibit considerable overlap, providing anatomical support for involvement of the gonadal steroids in the control of GTH secretion in fish.

Steroid negative feedback regulation of gonadotropin secretion has been demonstrated in several teleost species by classical gonadectomy/steroid replacement models. Elevations of blood GTH levels caused by gonadectomy in trout (Bommelaer et al., 1981), African catfish (Habibi et al., 1989) and goldfish (Kobayashi and Stacey, 1990) are suppressible by T or E_2 , and in the case of trout, also 11-keto-testosterone (11-KT), a non-aromatizable androgen found in teleost fish (Engel, 1975; Ozon, 1972). Implantation of antiestrogens into the brain and pituitary of intact goldfish stimulated GTH release (Billard and Peter, 1977), which has been interpreted as further evidence for steroid negative feedback; pituitary implantation, however, may have damaged the pituitary stalk and disrupted

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inhibitory dopaminergic inputs into the pituitary (Peter et al., 1986) and caused GTH release.

Evidence for the positive effects of gonadal steroids on GTH secretion comes primarily from studies of immature salmonids and immature European eels. Increased pituitary GTH content occured following intraperitoneal administration of T in juvenile male and female rainbow trout (Crim and Evans, 1979). The positive feedback effect of T is dependent on aromatization since this response was blocked by the aromatase inhibitor 1,4,6-androstatrien-3,17-dione (ATD; Crim et al., 1981). In addition, *in vivo* treatment with T enhanced GnRH-induced GTH secretion *in vitro* (Crim and Evans, 1980). In all of these studies, no changes in plasma GTH were noted. However, in subsequent experiments implantation of juvenile rainbow trout with T-containing silastic capsules resulted in GTH release to the circulation after 2 months (Crim and Evans, 1983). GnRH implants had no effect on plasma GTH levels, but when co-implanted with T, increased plasma GTH was observed after 1 month, suggesting a need for steroid priming of the pituitary for GnRH stimulation of GTH release in immature trout. Positive effects of E_2 on development of GTH cells have also been demonstrated in the pituitary of immature European eels (Olivereau and Olivereau, 1979). In other studies on immature European eels it has been shown that the increase in pituitary GTH levels due to E_2 treatment is dependent on dose (Dufour et al., 1983) and mediated by stimulation of pituitary mRNA for alpha-subunit glycoprotein hormone (Counis et al., 1987). A positive effect of gonadal steroids in adult teleost species has yet to be reported.

The goldfish is a seasonally breeding cyprinid fish usually spawning in the spring months. Seasonal cycles of gonadal size, blood steroid and GTH levels have been reported (Kagawa et al., 1983; Kobayashi et al., 1986a). Following spawning there is a decrease in gonadal size and blood steroid levels, concurrent with a decrease in blood GTH levels. During the autumn and winter gonadal recrudescence period, gonadal size and sex steroid levels increase to maximal levels at spawning. Furthermore, the *in vivo* GTH response to exogenous GnRH is maximal just prior to spawning, when gonadal size is maximal (Habibi et al., 1989). Given these observations, we hypothesized that increasing steroid levels during the gonadal recrudescence period may have a positive effect on GTH secretion in the adult goldfish. Since the feedback effect of estrogen could be mediated via conversion to catecholestrogens (CE) in the brain or pituitary (Timmers et al., 1989), we also examined the effects of 2-hydroxy-catechol-estradiol (2-OH- E_2) and 4-hydroxy-catechol-estradiol (4-OH- E_2) on GTH secretion. Evidence is presented that both T and E_2 exert a positive effect on GnRH-induced but not basal GTH levels in intact female and male goldfish.

Materials and Methods

Animals

Common or comet varieties of goldfish (*Carassius auratus)* weighing 15-40 g were purchased throughout the year from a commercial supplier (Grassyforks Fisheries; Martinsville, Indiana, U.S.A.). Prior to experimentation, fish were acclimated to 17° C and natural simulated photoperiods (Edmonton, Alberta; lat. 53.6°, long. 113.5°). All animals were fed twice daily with Ewos trout pellets supplemented with Tetramin flakes.

Steroid Treatment

Solid silastic pellets containing T, E_2, DHT, ATD or no steroid (Blank) were manufactured as previously described (Pankhurst et al., 1986) except that elastomere contained 100 mg/g of steroid and prepared pellets were vigorously washed for 10 minutes in saline before use. Fish received a steroid dose of 100 µg/g body weight. In one experiment (May 1989) sexually mature fish were treated with 100 µg/g and 400 µg/g T and E_2. Silastic capsules containing 250 µg 11-KT in castor oil (Lee et al., 1986) were used to administer this steroid. Under tricaine methanesuphonate anaesthesia, pellets and capsules were implanted intraperitoneally (i.p.) through a 2-3 mm incision in the body wall. Testosterone, E_2 and DHT were purchased from Sigma Chemical Co. (St.Louis, MO., U.S.A.) and, ATD and 11-KT was purchased from Steraloids Inc. (Wilton, N.H., U.S.A.)

Experimental Protocols

Effect of T or E2 implantation on GTH secretion throughout the seasonal reproductive cycle

At various times of the year, at 5 or 10 days following implantation fish were injected i.p. with either saline (0.6 % NaCl) or D-Ala^6-Pro^9-N-ethylamide-LHRH (Syndel Laboratories Inc., Vancouver, Canada; LHRH-A; 0.1 µg/g) for assessment of the *in vivo* GTH release response. The time course of the GTH response to LHRH-A and other GnRH analogues is well established in the goldfish (Peter, 1983; Peter et al., 1986). Blood samples were taken from anaesthetized fish by caudal puncture using 25 G needles 6 hours after injection; this sampling time was chosen because preliminary experiments (data not shown) indicated that maximal difference in response in control versus steroid-implanted animals was at 6 h. Blood was allowed to clot overnight (at 4° C), and serum was collected by centrifugation and kept frozen (-20° C) until hormone analysis.

At the termination of an experiment, fish were sacrificed and total gonad and body weights recorded. A gonadosomatic index (GSI) was calculated as gonad weight/ total body weight X 100. Pituitaries were removed from saline injected animals, sonicated in 1 ml radioimmunoassay buffer and stored frozen until hormonal content determination. To assess T or E_2 release *in vivo*, serum steroid levels in saline injected pellet-implanted animals were determined at several times during the year.

Involvement of aromatization in the action of androgens

To examine the involvement of T aromatization in mediating its actions on pituitary GTH secretion, male and female fish were implanted, as described, with aromatizable androgen (T), or non-aromatizable androgens (Engel, 1975), i.e. DHT and 11-KT, and the pituitary response to exogenous LHRH-A examined. In another experiment, males were implanted with ATD (100 and 300 µg/g) 2 days prior to T implantation. Pituitary responsiveness was assessed 5 days following T treatment.

Effect of catecholestrogens on GTH secretion in male goldfish

In one experiment, mature male goldfish (75-100 g; purchased as sexually mature males in March and were producing milt when tested in July) were injected 3 times at hourly intervals with 0.33 µg/g 2-OH-E_2 or 4-OH-E_2 or vehicle. Steroid was first dissolved in a solution of 0.1% ascorbic acid in 70% ethanol and suspended 1:10 in peanut oil. Blood was sampled immediately prior to the first injection (0 h) and 1 h after the last injection (3 h) to assess the effects of CE on basal GTH levels. In a second experiment, males were pretreated with 1 µg/g 2-OH-E_2 or vehicle 1 hour prior to LHRH-A (0.1 µg/g) or saline injection. Treatment with 2-OH-E_2 continued at hourly intervals until blood sampling at 4 hours following LHRH-A injection. The short term injection protocol was chosen since similar treatments in the prepubertal male rat decrease luteinizing hormone levels (Rodriguez-Sierra and Blake, 1982). Furthermore, in a pilot study (data not shown) we found that long term CE treatment (1 or 5 µg/g every 2 days for 3 injections) killed approximately 50 % of injected fish. Catecholestrogens were purchased from Sigma Chemicals.

Involvement of endogenous steroids in modulating pituitary GTH secretion

Since human chorionic gonadotropin (hCG) injection stimulates testosterone secretion in goldfish (Kobayashi et al., 1986b), sexually regressing male goldfish (75-100 g; purchased as sexually mature males in March and were producing milt when tested in October) were injected (i.p) with 0.2 µg/g human chorionic gonadotropin (hCG; Sigma

Chemicals, lot 18F-0277) or saline every 3 days for 39 days. Eighteen hours following the last hCG injection, fish were injected with LHRH-A or saline, and the pituitary GTH response assessed 6 hours later as previously described.

Radioimmunoassays (RIA)

Serum and pituitary GTH concentrations were determined using a double antibody RIA (Peter et al., 1984) specific for GTH-II (VanDerKraak et al., 1992). Rabbit anti-carp GTH-II antiserum was used at a final dilution of 1:220,000. Minimum detection limit (95% maximum binding) was approximately 1 ng/ml. All samples (10-50 μl) were assayed in duplicate and within and between assay coefficients of variation were <10%. The cross reactivity of the GTH primary antibody with hCG was tested in a dose range of 0.5-2000 ng/ml and was found to be negligible. Serum T and E_2 concentrations were determined by RIA after ether extraction (20 μl serum) as previously described (Kobayashi et al., 1987). Minimum detection limit of these assays was 0.3 ng/ml.

Statistical Analyses

Data were analysed using the least squares method of analysis of variance (AOV; SAS Institute, Cary, NC). Serum GTH values were not normally distributed and were log-transformed prior to 2-way AOV. Pituitary GTH content and GSI data were normally distributed and were not transformed prior to 1-way AOV. Post-hoc means comparisons were made between treatment groups using the least-squares means. Differences between means (n=7-16) were considered statistically significant if $p<0.05$.

Results

In blank implanted control animals both T and E_2 levels varied between the 5 dates examined (Table 2.1). Sexually regressed animals in October or July had T levels at or below assay detection limit (0.3 ng/ml); at other times of the year T levels were approximately 2 ng/ml. Sexually regressed animals in October or July had E_2 levels at or below detection limit; at other times of the year E_2 levels ranged from 0.6 to 1.9 ng/ml. Implantation of T or E_2 caused elevation of the respective steroid relative to blank implanted animals (Table 2.1). Levels of T and E_2 were physiological in that they were in the concentration ranges reported for ovulatory female goldfish (Kagawa et al., 1983; Kobayashi et al., 1986a; Kobayashi et al., 1987).

The effects of steroid treatments on GSI and pituitary GTH content are shown in Table 2.2. Maximal and minimal values for both GSI and pituitary GTH content were noted in the spring and summer months, respectively. Both GSI and pituitary GTH content were high in May 1988 and decreased progressively to minimum values in August. Both parameters increased throughout the autumn and winter months. Following spring spawning GSI and pituitary GTH content decreased again in June-July 1989. Treatment with either T or E_2 did not affect GSI or pituitary GTH content at any time of the year.

The effects of steroid implantation on serum GTH in saline-injected controls and LHRH-A injected female goldfish are presented in Table 2.3. Seasonal variations in serum GTH levels in blank implanted saline-injected animals were evident. There is an apparent correlation in the seasonal pattern in serum GTH levels with changes in GSI and pituitary GTH content; maximal and minimal serum GTH values were also noted in the spring and summer months, respectively. Treatment with either T or E_2 did not affect GTH levels in saline injected control animals at any time of the year. Injection of LHRH-A in blank implanted animals increased GTH levels in May 1988, and in October 1988 to May 1989, but not at other times of the year. Estradiol treatment potentiated the LHRH-A induced secretion of GTH in March 1988, June-October 1988 and June 1989, but not at any other time tested. Testosterone treatment clearly potentiated the effect of LHRH-A on serum GTH throughout the year. In males in January and July (Table 2.4) LHRH-A stimulated GTH secretion. Testosterone treatment did not affect GTH levels in saline injected control fish, but potentiated the effects of LHRH-A on GTH secretion. Furthermore, treatment with E_2 for 10 or 5 days did not affect serum GTH in saline injected males, but potentiated the LHRH-A effect on GTH secretion (Table 2.5).

Since E_2 did not and T effectively enhanced LHRH-A induced GTH secretion in sexually mature fish, we tested a higher steroid dose in these fish. Implantation of either 400 µg/g E_2 or T increased serum steroid levels to 15.2 ± 2.7 and 19.3 ± 3.2 ng/ml, respectively. In comparison to the 100 µg/g dose (see May 1989; Table 2.1), this was an approximate increase of 150 % for E_2 and 170 % for T. Testosterone levels were very similar to levels reported for ovulatory goldfish (Kobayashi et al., 1986a; 1987) whereas those of E_2 were higher than those seen at ovulation. The higher doses of steroid did not affect pituitary GTH content or GSI (data not shown). The effect of the higher dose of E_2 enhanced LHRH-A induced GTH secretion (Table 2.6). In contrast, the higher dose of T did not further enhance induced GTH secretion.

The effects of T and non-aromatizable androgens were tested in both females and males. In July (Table 2.7) LHRH-A did not stimulate GTH release in blank implanted females. In contrast, serum GTH levels were increased following LHRH-A injection in T-implanted animals. In this experiment, treatment with DHT had no effects on serum GTH levels in saline-injected animals and did not alter pituitary responsiveness to LHRH-A. There were no differences in pituitary GTH contents for blank, T and DHT treated fish (24.3±7.3, 19.0±2.2 and 19.3±4.0 µg/gland, respectively). In November (Table 2.7), LHRH-A did not stimulate GTH release in blank implanted females, but LHRH-A clearly stimulated GTH secretion in T-treated animals. Treatment with either 11-KT or DHT did not affect serum GTH levels in saline-injected females and did not potentiate the effect of LHRH-A on GTH secretion. In male goldfish tested in January (Table 2.8), LHRH-A stimulated GTH release in blank implanted animals and the response was potentiated by treatment with T. Implantation with either 11-KT or DHT did not affect serum GTH levels in saline-injected males and did not potentiate the stimulatory effect of LHRH-A on GTH secretion.

The effects of ATD (100 and 300 µg/g), an aromatase inhibitor, on the potentiating action of T on the GTH release response to LHRH-A are shown in Table 2.9. In blank implanted animals, LHRH-A stimulated GTH release and this response was potentiated in T treated males. In contrast, ATD alone at both doses did not affect serum GTH levels in saline-injected animals and did not affect the GTH response to LHRH-A. In fish pretreated with ATD and subsequently treated with T, serum GTH levels in saline-injected animals were slightly, but nonsignificantly reduced. At both doses ATD inhibited the potentiating effect of T on LHRH-A induced GTH secretion.

The effects of 3 injections of 0.33 µg/g 2-OH-E_2 and 4-OH-E_2 on basal GTH secretion in male fish are presented in Table 2.10. Injection with either catecholestrogen did not affect GTH levels. In addition, when the serum GTH data were expressed as a percentage of levels at time 0, no further effects were noted. In the second experiment, multiple injections of 1 µg/g 2-OH-E_2 did not affect basal or LHRH-A induced GTH secretion (Table 2.11).

The effect of injection of hCG every 3 days over a 39 day period on LHRH-A responsiveness is presented in Table 12. Injection of LHRH-A in saline treated control animals resulted in increased serum GTH levels. Treatment of males with hCG did not affect unstimulated serum GTH levels; however, hCG treatment significantly enhanced the GTH response to LHRH-A. Gonadal growth was not affected by hCG treatment; GSIs for control and treated groups were 3.8 ± 0.3 and 3.9± 0.3 %, respectively.

Discussion

Seasonal variations in GSI and serum levels of T and E_2, basal serum GTH levels, and pituitary GTH content were evident in the present study, and confirm previous reports (Kagawa et al., 1983; Kobayashi et al., 1986a). Seasonal variations in the response of serum GTH to exogenous GnRH parallel seasonal changes in gonadal size (Habibi et al., 1989). In the present experiments LHRH-A increased serum GTH from October to May, a period when gonadal size and serum steroid levels (Kagawa et al., 1983) are increasing. In sexually regressed animals (i.e. summer) with small GSIs, LHRH-A was ineffective in stimulating GTH secretion. With the exception of June 1988 when GSI was intermediate, E_2 enhanced the LHRH-A response in months when gonadal size was relatively low.

Estradiol was not effective in potentiating the GTH response to LHRH-A in the period of gonadal recrudescence from November 1988 to May 1989. These observations suggest that when gonadal size and serum E_2 levels are increasing naturally, exogenous E_2 does not further enhance LHRH-A induced GTH secretion. In the period immediately following the spawning season, fish are typified by having moderately large atretic ovaries and low blood sex steroid levels (Kagawa et al., 1983). In the present study, we found that post-spawning females in June 1988 had moderately sized atretic ovaries, moderately high pituitary GTH content and no response to LHRH-A. Furthermore, when these fish received E_2 treatment, LHRH-A effectively stimulated GTH release. In contrast, in 1989, fish came into spawning condition several weeks earlier than in 1988, and although most fish had atretic ovaries in May, the GSI was still relatively large and they were producing estradiol (> 1 ng/ml). These fish were responsive to LHRH-A and physiological doses of T, but not E_2, enhanced this response. In June 1989, post spawning fish had small ovaries and were not responsive to LHRH-A; however both T and E_2 effectively enhanced this response. Given that fish during the post-spawning period in different years exhibit highly variable gonadal size and condition, variable pituitary GTH contents and responses to LHRH-A, further investigation of the role of GnRH and gonadal steroids in the control of GTH secretion during this period of the reproductive cycle is warranted.

In contrast to E_2, implantation of T effectively enhanced the GTH response to LHRH-A throughout the entire seasonal reproductive cycle. The reason for the differential response of T and E_2 is unclear since we have demonstrated that T aromatization to estrogen is important for the positive effect of T on LHRH-A induced GTH secretion. Goldfish brain aromatase activity (Pasmanik and Callard, 1986) is maximal during the spawning season. Similarly, albeit to a lesser degree, pituitary aromatase also varies slightly throughout the year; lower activity being evident in the summer (Pasmanik and Callard, 1988). Given that T action is dependent on aromatase and that its effect is present in all seasons, T may be efficiently aromatized throughout the year and effectively increase local brain and pituitary E_2 concentrations above control levels. In contrast, elevation of serum E_2 by hormone implantation may have only increased tissue E_2 concentrations to effective levels in months when endogenous serum E_2 was low and not during months when endogenous E_2 and gonadal size were increasing. Implantation of female fish with a high dose (400 μg/g) of E_2 produced non-physiological serum E_2 levels that were approximately 2 times normal ovulatory concentrations (Kobayashi et al., 1986a; 1987). This resulted in no change in basal secretion but did enhance LHRH-A induced secretion. In contrast, both doses of T produced physiological serum T concentrations and enhanced LHRH-A induced GTH secretion. These data suggest that the lack of E_2 effect in some months is not a result of an inability to respond to estrogen treatment but rather that circulating serum E_2 levels are inadequate to elicit an effect. This does not seem to be the case for T since it can be converted efficiently to E_2 within the brain and pituitary of the goldfish (Pasmanik and Callard, 1988). Perhaps the affinity of tissue uptake or binding mechanisms favours T over E_2, resulting in a greater effectiveness of T in our experiments. One other possible explanation of the failure of E_2 to have an effect when T clearly enhanced GTH secretion may relate to the presence of serum steroid binding proteins. The characteristics of a T and E_2 binding globulin in goldfish serum have been described (Pasmanik and Callard, 1986). It has a high affinity that is similar for T and E_2 and the number of serum binding sites does not vary between sexes or seasons. These observations and the fact that the steroid-binding globulin interaction is highly unstable, Pasmanik and Callard (1986) suggest that the binding protein would not limit but enhance exchange of T and E_2 in neural tissues. It seems unlikely, therefore, that the steroid binding protein would decrease the effectiveness of steroid treatments. Further experiments will be necessary to clarify the basis for a seasonal variation in E_2 positive action.

Positive effects of sex steroids on GTH have been reported for several fish species (see Introduction). Testosterone and E_2 induce synthesis and accumulation of pituitary GTH in immature salmonid fish (Crim and Evans, 1979; Crim et al., 1981) and European eels (Dufour et al., 1983). Furthermore, Crim and Evans (1983) have demonstrated that long-term T treatment also stimulates GTH release into the blood of salmonids. They suggested that the pituitary GTH response to T in immature salmonids may be an example of steroid positive feedback. Indeed, two injections of salmon GTH stimulate dramatic increases in pituitary GTH content in immature male and female trout (Crim et al., 1982). Furthermore, the androgen, 11-KT can stimulate pituitary GTH accumulation (Crim et al., 1981; Weil and Marcuzzi, 1990) and affect pituitary responsiveness to GnRH (Weil and Marcuzzi, 1990) in trout . The lack of effect of 11-KT on pituitary GTH content, basal and LHRH-A induced GTH secretion in the goldfish suggests that this steroid acts differently in cyprinid and salmonid fish. In the present experiment steroid treatments did not affect pituitary GTH levels; therefore, the positive action of T and E_2 cannot be explained by steroid-induced increases in pituitary GTH. Mimicking increased GTH secretion by multiple hCG injection in male goldfish also resulted in enhanced GTH secretion in response to LHRH-A. This response is presumed to be mediated by stimulation of testicular steroidogenesis, since Kobayashi et al. (1986b) have demonstrated that hCG injection elevates blood levels of T in male goldfish. Ovariectomized or sexually regressed goldfish implanted with T or E_2 respond to environmental spawning cues (plants and increased water temperature) with an ovulation-like surge in serum GTH (Kobayashi et al., 1989). In the adult goldfish, there may be a dependence on endogenous gonadal steroids to prime the pituitary GTH system, therefore ensuring adequate GTH release and successful ovulation during the short spawning season exhibited by this species.

The mechanism and site of action of T and E_2 to increase pituitary responsiveness in the goldfish is unknown, but does not seem to involve accumulation of pituitary GTH. In other vertebrate species, sex steroids can act at the pituitary to reduce or enhance the GTH reponse to GnRH. In mammals, estradiol can either increase or decrease GnRH responsiveness by direct action on pitutary gonadotrophs (Kalra and Kalra, 1983; Frawley and Neill, 1984) whereas androgens have a direct action on the pituitary to inhibit gonadotropin secretion (Drouin and Labrie, 1976; Denef et al., 1980). In comparison, in the immature trout, both T and E_2 can act directly on the pituitary *in vitro* to enhance LHRH stimulated GTH release (Fahraeus-van Ree et al., 1983). In adult female trout, E_2 can also act direcly at the pituitary to enhance GnRH stimulated GTH secretion (Weil and Marcuzzi, 1990b). In goldfish, gonadal steroids also have a direct positive action on the pituitary since we have found that T (100 nM for 24 h incubation) increased the response of pituitary fragments to GnRH *in vitro* (see Chapter 3).

Very little is known about steroid feedback control of GTH secretion in other cold-blooded vertebrates. In gonadectomized ranid frogs, *in vivo* treatment with E_2 inhibits and DHT augments *in vivo* GnRH responsiveness (McCreery and Licht, 1984) and in the case of E_2 also *in vitro* GnRH responsivess (Pavgi and Licht, 1989). In a single study in a turtle (Licht, 1985), the same research group demonstrated that gonadectomy elevated and T and E_2 suppressed pituitary reponsivess to GnRH. In the case of goldfish, both T and E_2 (but not 11-KT or DHT) had a positive effect on LHRH-A induced GTH secretion *in vivo*. These differences in steroid action on GTH secretion may represent true species variations; however, the experimental protocols employed vary greatly between studies, making comparisons difficult. Regardless, it is clear that the relative importance of aromatase and reductase activity in androgen action varies amongst the vertebrates.

Gonadectomy results in elevated blood GTH, and steroid replacement therapy in gonadectomized teleosts reduces these elevations in GTH (Habibi et al., 1989; Kobayashi and Stacey, 1990). In the case of intact male and female goldfish, however, no negative actions of gonadal steroids were observed at any time of the year, suggesting that the steroid response in gonadectomized versus intact paradigm are quite different. The reason for this difference is not known.

It is possible that alterations in GnRH receptor numbers can account for the enhancement of GTH secretion following steroid treatment in the gonad-intact goldfish, since there is a good corelation between GnRH receptor binding and pituitary GnRH responsiveness in other vertebrate models (Clayton et al., 1985; Conn et al., 1987). In mice, gonadectomy reduces whereas E_2 treatment increases pituitary GnRH receptor levels; this is in contrast to rats where gonadectomy increases and E_2 suppresses pituitary GnRH receptor numbers (Clayton et al., 1985). In male catfish, castration increased and androstenedione suppressed pituitary GnRH receptor numbers, respectively (Habibi et al., 1989). Castration-induced increases in GnRH receptor number was accompanied by an increased GTH response to GnRH *in vivo* (Habibi et al., 1989). Although the interaction of sex steroids and GnRH receptor binding has not been tested in goldfish, pituitary responsiveness to GnRH, GnRH receptor number (Habibi et al., 1989) and serum gonadal steroid levels increase during seasonal reproductive development in this species. We suggest that the positive action of T and E_2 on GnRH-induced GTH secretion in the intact goldfish may also be related to changes in pituitary GnRH receptor numbers. This hypothesis is addressed in Chapter 3.

It is possible that gonadal steroids could act to modulate dopaminergic inhibition of GTH secretion. In rats, gonadal steroids act to affect hypothalamic catecholamine turnover and subsequently, GnRH and GTH secretion (Barraclough et al., 1984). In goldfish and other teleost species, dopamine (DA) has a strong inhibitory influence on GnRH-induced GTH secretion (Peter et al., 1986; 1988) and administration of various DA antagonists potentiates the actions of GnRH agonists on GTH secretion. The maximal increases in serum GTH in response to the DA antagonists pimozide (Sokolowska et al., 1985) and domperidone (Omeljaniuk et al., 1989), are greatest in sexually mature goldfish, suggesting that the DA inhibitory tone is greatest when gonadal function (i.e. sex steroid secretion) is maximal. *In vivo* domperidone dose response curves did not, however, reveal any differences in drug sensitivity (ED_{50}) throughout the reproductive cycle (Omeljaniuk et al., 1989). This suggests that factors other than alterations in the DA inhibitory tone are responsible for seasonality of DA antagonist action. Gonadal steroids may influence GTH secretion by altering catecholamine metabolism in the brain, since E_2 modulates hypothalamic monoamine oxidase (MAO) in goldfish (Olcese and de Vlaming, 1979) and Indian catfish (Manickam and Joy, 1989). Since steroid treatment effects on pituitary hormone secretion were not evaluated in these studies (Olcese and de Vlaming, 1979; Manickam and Joy, 1989), the physiological relevance of E_2 regulation of hypothalamic MAO activity is not known. The interaction of catecholamines and gonadal steroids in the control of GTH secretion has been addressed in Chapter 4. Using the male African catfish as a model, one research group has proposed (DeLeeuw et al., 1987; Timmers et al., 1989) that the negative feedback effects of androgen on GTH secretion are mediated by their conversion, within the brain and/or pituitary, to estrogens and subsequently to catecholestrogens (CE). They propose that CE compete with DA for catechol-O-methyl-tranferase and cause decreased DA degradation and hence increase inhibitory DA effects on GTH secretion. Our data in the goldfish clearly indicate that androgen and estrogen modulate the effect of GnRH on GTH secretion; however, our limited experiments have thus far failed to demonstrate similar effects for CE. Whether there is a fundamental difference in the mechanism of estrogen action on GTH secretion in the African catfish and goldfish remains to be resolved.

In conclusion, by using a gonad- intact, steroid-treated paradigm we have demonstrated for the first time that T and E_2 can enhance LHRH-A-induced GTH secretion in an adult teleost species; in the case of E_2, the effect varies seasonally. We suggest that elevations in endogenous steroid levels during gonadal recrudescence may prime the pituitary and ensure that changes in GnRH (Yu et al., 1987; Yu et al., 1991) and

GTH release, in response to environmental and pheromonal cues (Hontela and Stacey, 1990), result in successful spawning in this species.

Table 2.1. Serum estradiol and testosterone levels (ng/ml) in female goldfish at various times of the year (1988-1989). Data are presented as mean ±SEM (n=7-14).

	Treatment					
	Blank		Estradiol		Testosterone	
Date	E_2 level	T level	E_2 level	T level	E_2 level	T level
March 4/88	0.6±0.2	2.3±0.9	7.4±0.4	2.0±0.7	0.6±0.2	6.5±0.3
May 21	1.9±0.2	2.2±0.7	6.0±1.0	1.7±1.1	NP	NP
Oct. 27	0.3±0.1*	0.5±0.1	10.3±1.0	0.6±0.2	0.3±0.1*	8.8±0.5
May 8/89	1.2±0.3	2.4±0.4	10.5±1.1	1.0±0.1	0.6±0.1	11.1±1.5
July 18	0.3±0.1*	0.4±0.1	NP	NP	0.3±0.1*	14.4±2.3

NP- experiment not performed

* - Most samples in October and July were below E_2 assay detection limit (0.3 ng/ml) and were assigned this value

Table 2.2. Effects of estradiol and testosterone implantation on gonadosomatic index and pituitary GTH content in female goldfish at various times of the year (1988-1989). Data are presented as mean ±SE (n=7-14).

	Gonadosomatic Index (% BWt)*			Pituitary GTH (μg/gland)*		
Treatment	Blank	E_2	T	Blank	E_2	T
Date						
March4/88	NP	NP	NP	NP	NP	NP
May 21	7.3±1.9	8.0±2.1	NP	92.7±7.0	87.4±17.6	NP
June 4	5.2±1.5	4.3±1.2	NP	57.6±9.1	48.2±5.9	NP
July 19	1.8±0.2	1.3±0.1	NP	11.1±4.8	15.6±3.5	NP
August 9	1.2±0.1	1.3±0.1	NP	6.9±1.2	8.3±3.5	NP
October 27	3.0±0.3	2.2±0.2	2.5±0.2	31.6±4.8	35.9±3.7	31.8±3.9
Nov. 16	3.9±0.4	2.7±0.5	3.1±0.5	27.6±5.5	25.0±6.6	23.9±6.5
Feb 9/89	4.7±0.8	6.7±0.8	4.7±0.9	29.1±5.0	45.8±3.2	39.2±8.4
March 29	8.8±1.5	7.4±1.3	7.9±1.4	48.2±3.9	40.9±4.4	42.6±6.0
May 8	8.1±1.3	7.7±1.4	7.1±1.1	49.3±6.3	46.8±5.0	47.1±5.7
June 3	2.6±1.2	3.4±1.1	2.5±1.1	26.6±8.8	36.2±7.0	30.7±6.4
July 18	2.3±0.5	NP	1.8±0.1	24.3±7.3	NP	20.9±4.1

NP- experiment not performed

*-there was no effect ($p>0.05$) of steroid treatment on GSI or pituitary GTH content at any time of the year

Table 2.3. The effect of estradiol and testosterone implantation on serum GTH levels (ng/ml) in saline-injected (control) and LHRH-A (0.1 μg/g)-injected female goldfish at various times of the year (1988-1989). Data are presented as mean ±SE (n=7-14).

	Serum GTH (ng/ml)					
	Saline-Injected**			LHRH-A-Injected		
Treatment	Blank	E_2	T	Blank	E_2	T
Date						
Mar 4/88	7.4±0.9	4.9±1.2	5.0±1.0	21.3±8.9	42.0±7.9*	66.5±12.4†
May 21	16.7±2.9	16.9±4.5	NP	152.6±38≠	139.1±37.2	NP
June 4	9.6±1.8	7.9±1.6	NP	21.2±9.4	84.0±23.7*	NP
July 19	4.6±0.6	5.3±0.8	NP	5.3±0.8	17.1±5.6*	NP
August 9	3.6±0.2	5.0±0.5	NP	6.0±0.6	20.7±3.1*	NP
Oct. 27	4.6±0.5	3.9±0.5	5.5±1.4	20.7±5.1≠	40.4±8.1*	80.8±13.7 †
Nov. 16	5.9±1.2	4.3±0.5	5.1±0.7	49.5±10.9≠	40.9±9.6	94.5±17.6 †
Feb 9/89	11.0±1.7	10.8±2.4	7.8±0.5	82.6±21.9±	133.4±31.0	230.0±46.3†
March 29	11.9±0.9	7.5±1.4	8.0±1.0	61.4±18.9≠	56.7±11.1	169.9±27.7†
May 8	10.3±1.6	9.3±1.7	9.0±1.4	41.0±9.2≠	58.8±8.9	141.3±24.6†
June 3	4.4±1.1	5.7±1.5	5.6±1.0	4.9±2.0	84.0±33.7*	217.5±72.0†
July 18	4.5±1.3	NP	2.6±0.8	9.5±4.5	NP	39.3±2.0 †

NP- experiment not performed

**- there was no effect ($p>0.05$) of steroid treatment on unstimulated GTH levels at any time of the year

≠- significant ($p<0.05$) effect of LHRH-A versus saline-injected Blank-implanted animals

*- significant ($p<0.05$) enhancement of LHRH-A effect by E_2 versus LHRH-A injected Blank-implanted animals

†- significant ($p<0.05$) enhancement of LHRH-A effect by T versus LHRH-A injected Blank-implanted animals

Table 2.4. The effect of testosterone implantation on serum GTH (ng/ml) in saline-injected (control) and LHRH-A (0.1 µg/g)-injected male fish. Data are presented as mean ± SE (n=10-13).

		Serum GTH (ng/ml)	
	Treatment	Saline**	LHRH-A
Date			
Jan/89	Blank	3.3±0.9	16.8±6.0≠
	Testo	3.5±1.2	70.0±27.8†
July/89	Blank	6.4±1.0	26.7±5.7≠
	Testo	11.3±5.1	52.8±11.4†

**- there was no effect ($p>0.05$) of steroid treatment on unstimulated GTH levels

≠- significant ($p<0.05$) effect of LHRH-A versus saline-injected Blank-implanted animals

†- significant ($p<0.05$) enhancement of LHRH-A effect by T versus LHRH-A- injected Blank-implanted animals.

Table 2.5. The effect of estradiol implantation on serum GTH (ng/ml) in saline-injected (control) and LHRH-A (0.1 µg/g)-injected male fish. Data are presented as mean ± SE (n=7-8).

		Serum GTH (ng/ml)	
	Treatment	Saline**	LHRH-A
Date			
Nov/88	Blank	7.6±1.9	13.9±2.4≠
(10 days)	E_2	4.7±1.0	35.0±3.6*
August/88	Blank	3.3±0.5	8.0±4.7
(5 days)	E_2	5.9±0.6	19.4±5.3*

**- there was no effect ($p>0.05$) of steroid treatment on unstimulated GTH levels

≠- significant ($p<0.05$) effect of LHRH-A versus saline-injected Blank-implanted animals

*- significant ($p<0.05$) enhancement of LHRH-A effect by E_2 versus LHRH-A-injected Blank-implanted animals.

Table 2.6. The effect of testosterone (100 and 400 µg/g) and estradiol (100 and 400 µg/g) implantation on serum GTH (ng/ml) in saline-injected (control) or LHRH-A-injected female fish in May 1989. Data are presented as mean ± SE (n=9-16).

	Serum GTH (ng/ml)	
Treatment [(a)]	Saline**	LHRH-A
Blank	10.3 ± 1.6	41.0 ± 9.2≠
Testo (100)	9.0 ± 1.4	141.3 ± 24.6†
Testo (400)	7.7 ±0.8	93.9 ± 19.0†
E_2 (100)	10.5 ± 1.1	58.8 ± 8.9
E_2 (400)	10.8 ± 2.0	86.2 ± 22.0*

(a)- Data for Blank, Testo (100) and E_2 (100) are repeated from Table 2.3 for comparison.

**- there was no effect ($p>0.05$) of steroid treatment on unstimulated GTH levels

≠- significant ($p<0.05$) effect of LHRH-A versus saline-injected Blank-implanted animals

†- significant ($p<0.05$) enhancement of LHRH-A effect by T versus LHRH-A injected Blank-implanted animals

*- significant ($p<0.05$) enhancement of LHRH-A effect by E_2 (400 µg/g dose only) versus LHRH-A-injected Blank-implanted animals

Table 2. 7. The effect of various androgens on serum GTH (ng/ml) in saline-injected (control) and LHRH-A (0.1 μg/g)-injected female fish. Data are presented as mean ± SE (n=8-12).

		Serum GTH (ng/ml)	
Date	Treatment	Saline*	LHRH-A
July/89	Blank	4.5±1.3	9.4±4.5
	Testo	2.6±0.8	39.3±6.8†
	DHT**	2.3±0.5	7.5±2.0
Nov/89	Blank	7.8±1.5	13.4±4.2
	Testo	5.1±0.6	107.9±12.8†
	11-KT**	4.0±0.5	6.7±1.3
	DHT**	4.1±0.4	8.2±2.1

---

*- there was no effect ($p>0.05$) of steroid treatment on unstimulated GTH levels.

**- neither 11-KT nor DHT affected GTH levels in saline-injected animals and nor affected LHRH-A-induced GTH secretion ($p>0.05$).

†- significant ($p<0.05$) enhancement of LHRH-A effect by T versus LHRH-A-injected Blank-implanted animals

Table 2.8. The effect of various androgens on serum GTH (ng/ml) in saline-injected (control) and LHRH-A (0.1 µg/g)-injected male fish (Jan/90). Data are presented as mean ± SE (n=8-14).

Treatment	Serum GTH (ng/ml) Saline*	 LHRH-A
Blank	3.6±0.8	19.9±5.2≠
Testo	2.9±0.7	107.9±29.1†
11-KT**	2.5±0.5	12.1±2.8
DHT**	3.6±0.7	14.4±3.3

---

*- there was no effect (p>0.05) of steroid treatment on unstimulated GTH levels.

**- neither 11-KT nor DHT affected GTH levels in saline-injected animals and nor affected LHRH-A-induced GTH secretion (p>0.05).

≠- significant (p<0.05) effect of LHRH-A versus saline-injected Blank animals

†- significant (p<0.05) enhancement of LHRH-A effect by T versus LHRH-A-injected Blank-implanted animals.

Table 2.9. The effect of testosterone alone or with ATD (100 and 300 μg/g) pretreatment on serum GTH (ng/ml) in saline-injected (control) or LHRH-A (0.1 μg/g)-injected male fish in March 1990. Data are presented as mean ± SE (n=7-10).

	Serum GTH (ng/ml)	
Treatment	Saline**	LHRH-A
Blank	12.8±2.7	46.1±8.9≠
Testo	12.5±2.0	140.8±13.3†
ATD (100)	9.2±0.8	54.5±13.3
ATD (100) + Testo	7.4±1.9	71.1±17.7*
ATD (300)	8.9±2.2	42.2±9.8
ATD (300) + Testo	6.8±1.4	61.4±18.4*

**- there was no effect ($p>0.05$) of steroid treatment on unstimulated GTH levels

≠- significant ($p<0.05$) effect of LHRH-A versus saline-injected Blank-implanted animals

†- significant ($p<0.05$) enhancement of LHRH-A effect by T versus LHRH-A-injected Blank-implanted animals

*- ATD blocked the stimulatory effect of T on LHRH-A-induced GTH secretion.

ATD (100 and 300 μg/g) alone did not affect ($p>0.05$) unstimulated or LHRH-A-induced GTH secretion.

Table 2.10. The effect of multiple injection (0.33 μg/g hourly; 3 injections) of 2-hydroxy-estradiol and 4-hydroxyestradiol on serum GTH (ng/ml) in male goldfish. Data are presented as mean ±SE (n=10).

	Serum GTH ng/ml		
Treatment *	0 h	3 h	% Time 0
Vehicle	9.2 ± 1.5	8.2 ± 2.0	81.7 ± 7.6
2-OH-E_2	10.1 ± 1.4	7.4 ± 1.5	69.6 ±7.2
4-OH-E_2	11.5 ± 1.9	10.6 ± 1.9	91.8 ± 8.8

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*- there was no effect ($p>0.05$) of catecholestrogen treatment on serum GTH levels or on % of Time 0 values.

Table 2.11 . The effect of multiple injections of 2-OH-E_2 on serum GTH (ng/ml) in unstimulated (saline-injected) or LHRH-A (0.1 μg/g)-injected male fish. Data are presented as mean ± SEM (n=10).

	Serum GTH (ng/ml)	
Treatment *	Saline	LHRH-A
Saline	20.7 ± 4.1	47.2 ± 13.2≠
2-OH-E_2	17.7 ± 2.1	28.5 ± 6.0

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* - there was no effect ($p>0.05$) of 2-OH-E_2 treatment on GTH levels in saline or LHRH-A injected animals.

≠- significant ($p<0.05$) effect of LHRH-A versus saline-injected animals.

Table 2.12. The effect of multiple hCG (0.2μg/g every 3 days for 39 days) injections on serum GTH (ng/ml) in unstimulated (saline-injected) or LHRH-A (0.1 μg/g)-injected male fish in October 1989. Data are presented as mean ± SEM (n=8-10).

	Serum GTH (ng/ml)	
Treatment	Saline	LHRH-A
Saline	7.8±1.0	27.3±3.6≠
hCG	8.3±0.9	48.9±4.5†

**- there was no effect ($p>0.05$) of hCG treatment on unstimulated GTH levels

≠- significant ($p<0.05$) effect of LHRH-A versus saline-injected control animals

†- significant ($p<0.05$) enhancement of LHRH-A effect by hCG injection versus LHRH-A-injected saline control animals

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3. EFFECTS OF SEX STEROID TREATMENTS ON SALMON GONADOTROPIN-RELEASING HORMONE AND CHICKEN GONADOTROPIN-RELEASING HORMONE-II STIMULATED GONADOTROPIN SECRETION FROM THE GOLDFISH PITUITARY

Introduction

Gonadal steroids are important feedback regulators of hypothalamo-hypophyseal function in vertebrates. Best studied are the actions of androgens and estrogens in the control of luteinizing hormone (LH) release in homeothermic vertebrates such as laboratory rodents (Kalra and Kalra, 1983; Fink, 1988), large ruminants such as the sheep (Karsh, 1987) and the domestic chicken (Sharp, 1983). The role of gonadal steroids in the control of gonadotropin (GTH) release in poikilothermic species, however, is not well understood.

Steroid negative feedback regulation of GTH release has been demonstrated in several teleost species by classical gonadectomy/steroid replacement paradigms (Bommelaer et al., 1981; Habibi et al., 1989b; Kobayashi and Stacey, 1990). Similarly, steroid negative feedback has been demonstrated in the frog (Pavgi and Licht, 1989) and turtle (Licht, 1985). Evidence for the positive action of gonadal steroids in cold-blooded vertebrates comes primarily from studies of immature salmonids (Crim and Evans, 1979; 1983) and immature European eels (Dufour et al., 1983) where testosterone (T) and estradiol (E_2) stimulate accumulation but not release of pituitary GTH.

We have recently established in adult, gonad-intact goldfish that sex steroids play an important role in the positive feedback control of GTH secretion (Trudeau et al., 1991). Testosterone, via aromatization to E_2, potentiates the serum GTH response to a synthetic long acting mammalian GnRH analogue ([D-Ala^6-Pro^9-N-ethylamide]-LHRH; LHRH-A), without affecting basal serum GTH levels. In the present series of experiments we sought to determine whether the effect of *in vivo* steroid treatment is maintained *in vitro* in the absence of supplemental E_2 and T.

Goldfish brain and pituitary contain two endogenous GnRH molecules, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II), which have been shown to stimulate GTH release *in vivo* and *in vitro* (Peter et al., 1987; Yu et al., 1988; Peter et al., 1990). We also sought to determine whether the potentiating effects of sex steroids are seen only with the synthetic long acting analog LHRH-A (Trudeau et al., 1991) or also with the endogenous GnRH molecules. To define whether T has a direct action at the pituitary to increase GnRH responsiveness, *in vitro* studies using pituitary fragments in perifusion were performed. The effects of *in vivo* steroid treatments on pituitary GnRH receptor binding and serum GTH response to GnRH were also assessed since in goldfish increased *in vivo* GTH response to GnRH is highest during maximum gonadal recrudescence, when sex steroid levels and GnRH receptor numbers are high (Habibi et al., 1989b).

Materials and Methods

Animals

Common or comet varieties of goldfish (*Carassius auratus*) were purchased throughout the year from commercial suppliers (Grassyforks Fisheries; Martinsville, IND, or Ozark Fisheries; Stoutland, MO, U.S.A.). Prior to experimentation, fish were acclimated to 17^{o}C, fed and maintained as previously reported (Trudeau et al., 1991).

Steroid Treatments *in vivo*

Solid silastic pellets containing T or E_2 (elastomer contained 100 mg/g of steroid) or no steroid (Blank) were manufactured as previously described (Chapter 2; Trudeau et al., 1991). Fish received a steroid dose of 100 µg/g body weight. Under tricaine methanesuphonate anaesthesia, pellets were implanted intraperitoneally (i.p.) through a 2-3 mm incision in the body wall. Testosterone and E_2 were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.).

Experimental Protocols

Effects of in vivo E_2 and T treatment on sGnRH- and cGnRH-II-stimulated GTH release in vitro

The effects of *in vivo* E_2 and T treatment on *in vitro* hormone release from pars distalis (p.d.) fragments of the goldfish pituitary were examined using an established perifusion system (Marchant et al.,1989).

In experiment 1, the effect of *in vivo* E_2 on GnRH-induced secretion *in vitro* was examined in sexually regressed female fish in July (GSI= 2 %). Females were implanted for 5 days with E_2 or control (blank) solid silastic pellets. Following dissection, pars distalis fragments were preincubated in perifusion for 2 hours prior to experimentation. Fragments were exposed at hourly intervals to 2-minute pulses of 0.5, 5 and 50 nM sGnRH and cGnRH-II. Medium effluent was collected at 5 minute intervals. The mean GTH levels of the 3 samples (15 minutes) preceeding a GnRH pulse were considered representative of basal secretion. A second experiment examining the effects of E_2 was carried out using sexually recrudescent female fish in October (GSI= 3 %) and the doses of sGnRH and cGnRH-II tested were 1, 10, and 100 nM. The effects of T were investgated using sexually recrudescent female fish in December (GSI= 5%) and the doses of sGnRH and cGnRH-II tested were 1, 10, and 100 nM.

The effects of E_2 and T were also tested using female fish in post-spawning condition in June (GSI= 3.5 %) and the doses of sGnRH and cGnRH-II tested were 0.1, 1, 10, and 100 nM. Finally, the effects of E_2 and T were examined in sexually regressed fish (pituitaries of both sexes pooled) in September (GSI= 1 %; the doses of sGnRH and cGnRH-II tested were 0.1, 1, 10, 100, and 1000 nM sGnRH.

Effects of T on GTH release in vitro

The effects of *in vitro* T treatment were examined using whole pituitary fragments of sexually regressed goldfish (pituitaries of both sexes pooled) in October (GSI= 1.7 %). In a pilot study conducted with sexually mature fish in May, 100 nM T and E_2 for 24 h *in vitro* enhanced the GTH response to a single 100 nM pulse of sGnRH (data not shown). Therefore, fragments were incubated with 100 nM T (initially dissolved in 98% ethanol; final ethanol concentration was $\leq 0.01\%$ v:v) or ethanol vehicle for 24 h in the presence or absence of 2.5×10^{-5}M cycloheximide, a protein synthesis inhibitor. Cycloheximide was administered 2 h prior to either ethanol or T. A full dose response curve for sGnRH (0.1-1000 nM) was determined.

Effects of T on pituitary GnRH receptor binding and GTH release

The effects of *in vivo* T treatment on pituitary GnRH receptor binding characteristics were assessed in sexually regressed goldfish (pituitaries of both sexes pooled). Fish were implanted with T or blank and pituitaries removed 5 days later. Preparation of goldfish pituitary membranes, iodination of the potent salmon GnRH analogue, [D-Arg^6, Trp^7, Leu^8, Pro^9]-N-ethylamine-GnRH (sGnRH-A) and receptor binding assay procedures were carried out as described by Habibi et al., (1987; 1989a). Purification of ^{125}I-sGnRH-A was achieved by using an HPLC system using a modification of the procedures described by Yu et al., (1987). The iodination mixture was injected via a 200 µl injection loop into an Econosil C18 (5 µ; Alltech Associates, Inc.) column with RCSS-C18 column guard (Waters Associates). The mobile phase was 24% acetonitrile in 0.25 M formic acid adjusted to pH 6.5 with triethylamine. The flow rate was 0.8 ml/min and column outflow was collected every 1 min. The reference standard for sGnRH-A was detected by UV absorbance (254 nm); sGnRH-A eluted approximately 30 min prior to ^{125}I-sGnRH-A. Specific activity of this preparation, determined by a self-displacement radioimmunoassay, was 1200 µCi/mg and was similar to that originally reported by Habibi et al. (1987).

Studies on the effects of T implantation on pituitary GTH responsiveness to sGnRH-

A were carried out concurrently with GnRH receptor binding experiments. In one experiment, sexually regressed male and female goldfish were implanted with T and the serum GTH response to i.p injection of sGnRH-A (0.01 µg/g; 5 µl/g; 0.6 % NaCl vehicle) was assessed 5 days later. Blood was collected from anaesthetized fish 6 h after sGnRH-A injection. In a second experiment, the effects of *in vivo* T treatment (5 days implantation) on the *in vitro* responsiveness of whole pituitary fragments to increasing doses (0.01-10 000 nM) of sGnRH-A were also determined. Pituitary sensitivity was assessed by estimation of ED_{50} values for sGnRH-A induced GTH release.

Effects of in vivo E_2 and T treatment on brain and pituitary sGnRH content and on in vitro sGnRH release from pituitary fragments in vitro

Since changes in GnRH responsiveness could be a result of homologous receptor regulation (Habibi, 1991), and sex steroids may affect GnRH production (Kalra and Kalra, 1983; 1989), the effects of 5 day implantation of T and E_2 on total brain and pituitary sGnRH contents were determined in sexually regressed female goldfish. On the day of experimentation, anaesthetized fish were killed by spinal transection and brains rapidly removed and placed on an ice-cold petri dish for dissection of the telencephalon including preoptic area (TEL-POA), hypothalamus (HYP) and pituitary (PIT) for determination of sGnRH contents. In teleost fish GnRH neurons directly innervate the pituitary (Kah, 1986); therefore, GnRH release from neuron terminals in pituitary fragments can be studied directly *in vitro* (Yu et al., 1991). The effects of *in vivo* steroid implantation on basal and 60 mM K^+-stimulated sGnRH release *in vitro* from pituitary fragments of sexually regressed goldfish was determined. Preparation of tissues and incubation procedures has been previously reported in detail (Yu et al., 1991). Briefly, pituitaries were removed, sliced and preincubated for 45 min before exposure to control and depolarization medium for 30 min. The equivalent of two pituitaries (in 500 µl) per incubation well (1 ml) was used. Medium and remaining tissue from control and K^+-exposed fragments were frozen for determination of sGnRH concentrations.

Radioimmunoassays (RIA)

Gonadotropin (GTH) concentrations were estimated using a double antibody RIA (Peter et al., 1984) specific for GTH-II (Van Der Kraak et al., 1992). Concentrations of sGnRH in brain, pituitary and incubation medium were estimated by a double antibody RIA similar to that reported by Yu et al., 1991. The rabbit anti-sGnRH antibody (S-30-3; generously donated by Dr. R. DeLeeuw, Organon Pharmaceuticals, The Netherlands) was used at a final dilution of 1: 100 000. Minimum detection limit of this assay (90 % B/Bo) was 0.5 pg/tube. Within and between assay coefficients of variation were < 10%. Cross-reactivity with cGnRH-II in the concentration range of 10-5000 ng/ml was 1.7 ± 0.3 % (n=11).

Statistical Analyses

GTH secretion *in vitro* was quantified as total amount of hormone (ng) released above basal hormone levels (sex steroid treatments *in vivo* did not affect basal GTH release *in vitro* at any time of the year). An increment of GTH following GnRH application was included as part of a response if it was greater than 1 S.E.M above basal secretion. Following GnRH application, GTH levels typically returned to basal within 10-15 min. Perifusion and serum GTH data were analysed by 2-way analysis of variance (SAS; Statistical Analysis Systems, Inc., Cary, NC). Means were considered statistically different if $p<0.05$. Dose response data for sGnRH-A were analysed using the Allfit computer program (DeLean et al., 1978). Estimates of ED_{50} were compared statistically using 95% confidence intervals. Scatchard analysis was carried out using the Ligand computer program (Munson and Rodbard, 1980) as validated for goldfish pituitary GnRH receptor binding (Habibi et al., 1987; 1989a).

Results

The effects of *in vivo* E_2 treatment on sGnRH- and cGnRH-II-stimulated GTH secretion from p.d. fragments of sexually regressed or early recrudescent female goldfish are shown in Figs. 3.1 and 3.2, respectively. Pars distalis fragments of blank implanted sexually regressed fish released GTH in response to 50 nM, but not to lower dosages of sGnRH and cGnRH-II (Fig. 3.1). In contrast, sGnRH and cGnRH-II stimulated GTH release in a dose-dependent manner from p.d. fragments of E_2-treated fish. Total GTH release in response to 5 and 50 nM sGnRH was significantly higher ($p<0.05$) from p.d.fragments of E_2-treated fish compared to controls. Total GTH release in response to all doses of cGnRH-II was significantly higher ($p<0.05$) from p.d. of E_2-treated fish compared to controls. sGnRH and cGnRH-II stimulated GTH release in a dose-dependent manner from p.d. fragments of sexually recrudescent goldfish (Fig. 3.2). Total GTH release in response to all doses of sGnRH and cGnRH-II was significantly higher ($p<0.05$) from p.d. of E_2-treated fish.

The effects of *in vivo* T treatment on sGnRH- and cGnRH-II-stimulated GTH release from p.d. fragments of sexually recrudescent female goldfish are shown in Fig. 3.3. sGnRH and cGnRH-II stimulated GTH release in a dose-dependent manner from p.d. fragments of blank-implanted fish. Total GTH release in response to all dosages of sGnRH and cGnRH-II was significantly higher ($p<0.05$) from p.d. of T-treated fish.

The effects of *in vivo* E_2 and T treatment on sGnRH- and cGnRH-II- stimulated GTH release from p.d. fragments of female goldfish in post-spawning condition are shown in Fig. 3.4. sGnRH and cGnRH-II stimulated GTH release in a dose-dependent manner from p.d. fragments of blank-implanted fish. The GTH release response to sGnRH was not potentiated by E_2- and T-pretreatment. Similarly, the GTH release response to cGnRH-II was not affected by E_2-pretreatment, however, total GTH release in response to 10 and 100 nM cGnRH-II was significantly higher ($p<0.05$) from p.d. of T-treated fish. The amount of GTH released in response to all doses of cGnRH-II was higher ($p<0.05$) than the amount of GTH released in response to sGnRH.

The effects of *in vivo* E_2 and T treatment on sGnRH- and cGnRH-II-stimulated GTH release from p.d. fragments of sexually regressed goldfish are shown in Fig. 3.5. sGnRH stimulated GTH release in a dose-dependent manner from p.d. fragments of blank-implanted fish, although, the release response was variable. In general, the GTH response to sGnRH was enhanced by E_2-pretreatment but was significantly different ($p<0.05$) from controls only at 100 and 1000 nM sGnRH. In contrast, pretreatment with T clearly potentiated ($p<0.05$) the GTH response to all doses of sGnRH. cGnRH-II stimulated GTH release in a dose-dependent manner from p.d. fragments of blank-implanted fish. Pretreatment with either E_2 or T clearly enhanced ($p<0.05$) the GTH release response to all doses of cGnRH-II. The amount of GTH released in response to 1000 nM cGnRH-II was higher ($p<0.05$) than the amount of GTH released in response to 1000 nM sGnRH.

The effects of *in vitro* T-treatment on sGnRH stimulated GTH secretion from whole pituitary fragments of sexually regressed goldfish are shown in Fig. 3.6. sGnRH stimulated GTH release in a dose-dependent manner from pituitary fragments exposed to ethanol vehicle. Exposure to 100 nM T for 24 h potentiated ($p<0.05$) the total ng GTH released in response to 1-1000 nM sGnRH. sGnRH stimulated GTH release in a dose-dependent manner from p.d. fragments exposed to cycloheximide. Although the GTH response to cycloheximude-exposed fragments was compared to controls, the differences were not significant. Co-treatment of pituitary fragments with cycloheximide and T abolished the potentiating effect of T on sGnRH-induced GTH release; GTH release-response data were similar to that in control and cycloheximide-treated pituitary tissues.

The effects of *in vivo* T-treatment on the serum GTH response to sGnRH-A are shown in Fig. 3.7. sGnRH-A stimulated a small but significant ($p<0.05$) increase in serum GTH in controls and T clearly potentiated ($p<0.05$) this response. The effects of T-implantation on *in vitro* GTH responsiveness are shown in Fig.3.8. sGnRH-A stimulated GTH release in a dose-dependent manner from control pituitaries and pre-exposure to T *in vivo* clearly enhanced the GTH response to 0.1-10 000 nM sGnRH-A. The ED_{50} estimates for GTH responses in control and T-treated groups were 1.0 ± 0.1 and 0.1 ± 0.1 nM ($p<0.05$), respectively. T treatment did not affect estimates of the equilibrium association constants of the high or low affinity receptor sites nor did it affect GnRH receptor binding capacity (Table 3.1). Treatment with T or E_2 did not affect total immunoreactive sGnRH content in the brain or pituitary or basal serum GTH levels in sexually regressed female goldfish (Table 3.2). Furthermore, basal and depolarization-induced sGnRH release from pituitary fragments *in vitro* was not affected by T and E_2 implantation (Table 3.3).

Discussion

Exposure of goldfish pituitary fragments *in vitro* to pulses of sGnRH, cGnRH-II and sGnRH-A results in dose dependent release of GTH, confirming previous studies with these peptides (Peter et al., 1987; Habibi et al.,1989a; Marchant et al., 1989). Pituitary fragments from sexually recrudescent fish released somewhat more GTH in response to low doses of either GnRH than did pituitary fragments from sexually regressed fish. This is in agreement with *in vivo* studies, in which it has been shown that the serum GTH responses to exogenous GnRH analogues are greatest when gonadal size and serum sex steroid levels are greatest (Sokolowska et al., 1985; Habibi et al., 1989b; Trudeau et al., 1991).

The *in vivo* implantation protocol employed in the present series of experiments elevates serum T and E_2 levels to values similar to those at ovulation in goldfish (Kobayashi et al., 1987; Trudeau et al., 1991). Such treatments do not affect basal GTH levels but enhance the serum GTH responses to LHRH-A (Trudeau et al., 199; Chapter 2) and sGnRH-A (present study). The positive effects of *in vivo* steroid treatment are maintained *in vitro* in the absence of supplemental E_2 and T. This effect is seen with both sGnRH and cGnRH-II, the endogenous GnRH molecules in the goldfish brain and pituitary (Yu et al., 1988). In one experiment using pituitary fragments from goldfish with post-spawning atretic ovaries, the positive effect of T was noted for cGnRH-II but not sGnRH-induced GTH release; moreover, estradiol was ineffective in changing GnRH responsiveness in this experiment. Similarly, T but not E_2 increases LHRH-A stimulated GTH release in post-spawning (i.e. sexually regressing) female goldfish *in vivo* (Trudeau et al., 1991; Chapter 2). It was also found in the same study that *in vivo* exposure to E_2 increased the serum GTH response to LHRH-A in sexually regressed but not sexually mature female goldfish; in contrast T is effective throughout the entire seasonal reproductive cycle.

The present studies also demonstrated that direct exposure of pituitary fragments to T enhances GnRH responsiveness. The level of T used in this experiment is physiological in that it approximates peak serum T concentrations on the day prior to ovulation in goldfish (Kobayashi et al., 1987). Testosterone action is protein synthesis-dependent, since co-treatment with the translation inhibitor, cycloheximide, blocked the potentiating effect of T on GnRH-stimulated GTH release. We hypothesized that the positive action of sex steroids on GnRH responsiveness may also involve increases in pituitary GnRH receptor number since this has been shown in other vertebrate models (Clayton et al., 1985; Conn et al., 1987; Gregg and Nett, 1989). However, we found that *in vivo* T implantation did not affect pituitary GnRH affinity or receptor number, even though the responsiveness of pituitary fragments to sGnRH-A was increased 10-fold by prior exposure to T. Furthermore, in the same stock of sexually regressed fish, *in vivo* responsiveness to sGnRH-A was enhanced 7-fold by T implantation. This is in contrast to rats (Drouin and Labrie, 1976; Kalra and Kalra; 1983) where androgens generally inhibit gonadotroph cell responsiveness to GnRH. The lack of effect of T on GnRH receptor binding was not expected since seasonal increases in GnRH responsiveness are associated with changes in GnRH receptor number (Habibi et al., 1989). Furthermore, castration in male African catfish increases pituitary responsiveness to GnRH peptides and also increases GnRH receptor binding capacity (Habibi et al., 1989). The effects of sex steroids seem to involve upregulation of the GTH release response to GnRH without changes in GnRH receptor number, brain and pituitary GnRH contents or changes in the amount of GnRH released from nerve terminals in the pituitary in response to K^+.

In other vertebrates, positive and negative feedback effects of steroids on gonadotropin secretion may not always be associated with changes in GnRH receptor binding (Clayton et al., 1985; Conn et al., 1987; King et al., 1989). Recently, E_2 has been shown to affect the release of LH by actions on intracellular signal transduction mechanisms. Liu and Jackson (1988; 1990) indicate that E_2 augments GTH release from rat anterior pituitary cells by interacting with Ca^{2+} and/or phospholipase C but not arachidonic acid mediated secretory mechanisms. Furthermore, E_2 action may also involve changes in protein kinase C activity (Audy et al., 1990). However, GnRH receptor binding studies were not performed in these experiments to enable evaluation of the relative contribution of receptor versus post-receptor mechanisms. In contrast, King et al., (1989) studied the negative feedback effects of several steroids in association with GnRH receptor studies. Testosterone, E_2 and progesterone all inhibited GnRH-induced LH secretion from cultured chicken pituitary cells by interacting with Ca^{2+} mobilization mechanisms. These effects were independent of changes in pituitary GnRH binding. Together, these studies indicate, that in addition to alterations in GnRH receptor number, sex steroids can affect gonadotropin release at multiple sites distal to GnRH receptor activation. In goldfish, sGnRH and cGnRH-II can compete for the same class of high affinity, low capacity receptor sites to stimulate GTH release (Habibi et al., 1989a; Cook et al., 1991). However, there are major differences in the intracellular pathways these two peptides activate; GTH release induced by sGnRH is slightly lower than that induced by cGnRH-II (Chang et al., 1990) and this was confirmed in several experiments presented here. cGnRH-II action is more dependent on extracellular Ca^{2+} entry than is sGnRH action (Jobin and Chang, 1990). Furthermore, sGnRH action involves activation of arachadonic acid metabolism whereas cGnRH-II action is independent of this signal transduction pathway (Chang et al., 1991). We do not know if gonadal steroids affect GnRH action in goldfish by affecting one or all of these pathways; however, the fact that T, and in some cases E_2, increased cGnRH-II stimulated GTH release to a greater extent than sGnRH-stimulated release is suggestive of such actions.

Sex steroid modulation of GTH secretion in goldfish may also involve changes in GTH synthesis as has been shown for other vertebrate species (Counis et al., 1987; Gharib et al., 1990; Mercer 1990). However, *in vivo* treatments with T and E_2 in gonad-intact (Trudeau et al., 1991) or ovariectomized (Kobayashi et al., 1990) adult goldfish do not affect the content of immunoreactive pituitary GTH. Sex steroid action in rats may also involve changes in glycosylation of gonadotropin molecules (Ramey et al., 1987; Krummen and Baldwin, 1988; Liu and Jackson, 1990) which could affect intracellular distribution of processed hormone and contribute to differential release of stored versus newly synthesized LH (Mukopadhyay et al., 1979; Ramey et al., 1987; Krummen and Baldwin, 1988). In the goldfish, sex steroids may also act to regulate GTH carbohydrate content and cellular distribution without changes in total immunoreactive pituitary GTH. Such changes could be another possible mechanism for potentiation of the GTH release response and could be expected to affect the biological activity of secreted GTH (Marut et al., 1981). Studies designed to address the effects of sex steroids on GTH biochemistry would contribute greatly to our understanding of positive feedback in this vertebrate group.

In summary, the present studies demonstrate that in gonad-intact goldfish, *in vivo* treatment with T and E_2 potentiates GnRH-induced GTH secretion *in vitro*. Testosterone has a direct effect on the pituitary to increase GnRH responsiveness. The potentiating effect of T on responsiveness to GnRH peptides is protein synthesis dependent, but does not involve changes in pituitary GnRH receptor affinity or number. Although the mechanisms of sex steroid positive action are unclear, it is likely that increases in serum sex steroid levels during seasonal gonadal development (Kobayashi et al., 1986) and ovulation (Kobayashi et al.,1987; 1989) enhance pituitary responsiveness to GnRH and may serve to coordinate the GTH surge (Aida, 1988; Kobayashi et al., 1989) at the time of spawning in goldfish.

Table 3.1. The effects of 5-day implantation of T (100μg/g) on equilibrium association constant (affinity; K_a) or binding capacity (R) of the high and low affinity pituitary GnRH binding sites. Binding characteristics (± SE) were estimated from Scatchard analysis of 4 individual displacement curves (13 concentration points, each incubated in triplicate). For each displacement curve, membrane fractions were prepared from 50 pituitaries from sexually regressed goldfish (sexes pooled).

	Blank	**Testo**
High affinity site		
K_a (10^{10} M^{-1})	1.1 ± 0.1	0.9 ± 0.1
R (fM/mg protein)	82.5 ± 16.7	86.4 ± 17.2
Low affinity site		
K_a (10^{7} M^{-1})	2.5 ± 0.8	2.3 ± 0.5
R (pM/mg protein)	24.8 ± 9.3	15.4 ± 5.7

Table 3.2. The effects of 5 day implantation of T and E_2 on brain and pituitary sGnRH contents (pg/tissue) and basal serum GTH (ng/ml) levels in sexually regressed female goldfish. Data are mean ± SEM (n=7-9).

	Blank	**Testo**	**Estradiol**
TEL-POA	1228 ± 248	808 ± 146	710 ± 88
HYP	630 ± 133	372 ± 77	513 ± 50
PIT	295 ± 54	538 ± 181	592 ± 141
Serum GTH (ng/ml)	4.3 ± 0.6	4.7 ± 0.8	3.4 ± 0.5

Table 3.3. The effects of 5 day implantation of T and E_2 on *in vitro* sGnRH release from pituitary fragments of sexually regressed goldfish. Data are the mean ± SEM (n=4).

	Blank	**Testo**	**Estradiol**
Medium (pg/ml)			
Control	8.7 ± .8	9.8 ± .9	12.8 ± 2.6
K^+	28.1 ± 9.5*	22.0 ± 1.7*	20.4 ± 2.7*
Tissue content (pg)			
Control	109.1 ± 9.8	155.4 ± 17.8	210.1 ± 95.2
K^+	166.2 ± 23.0	176.8 ± 25.3	145.1 ± 16.1

(*)- $p<0.05$ versus control

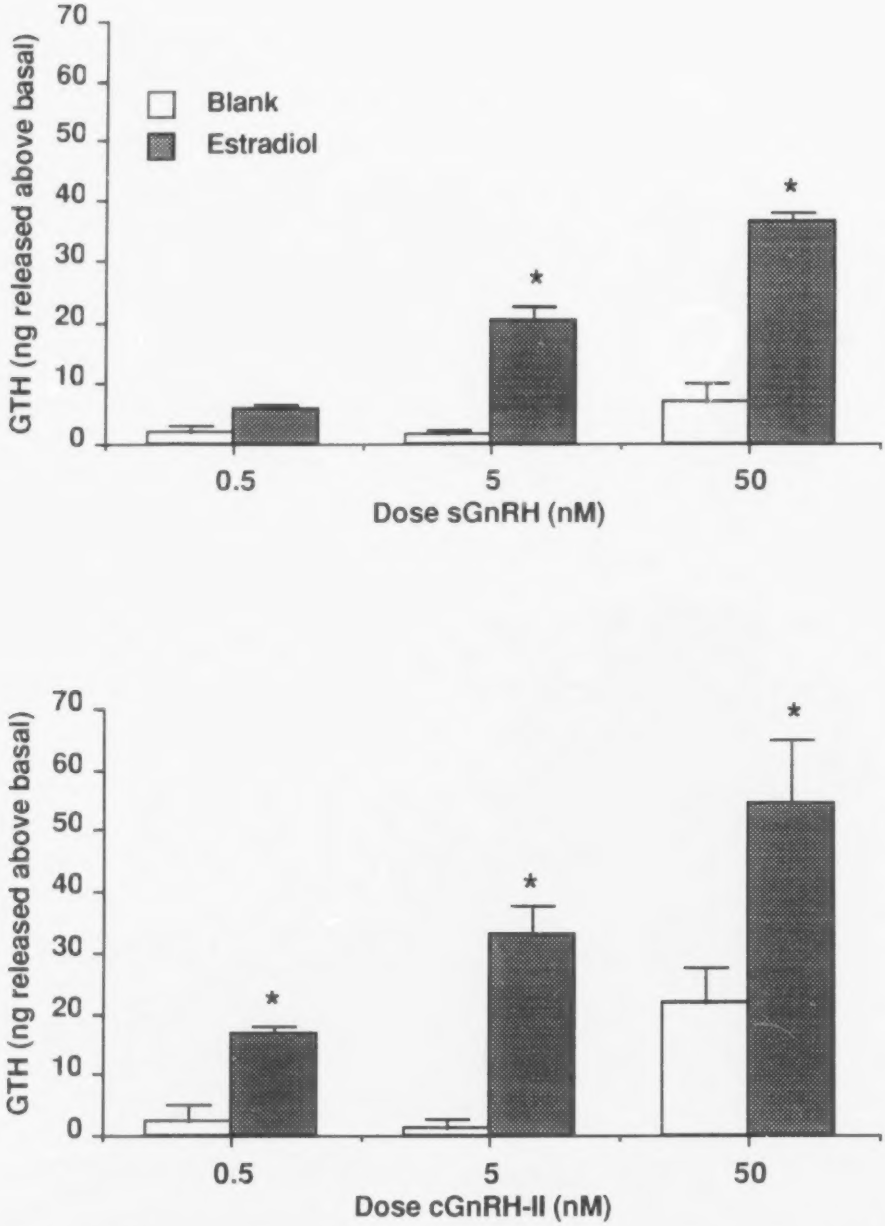

Fig. 3.1. The effects of intraperitoneal implantation of E_2 on the *in vitro* GTH release response to sGnRH (upper panel) and cGnRH-II (lower panel) in sexually regressed female goldfish. Data are the mean ± SE of the total ng GTH released above basal. (n=3 columns for sGnRH and n=2 columns for cGnRH-II). The (*) indicates a significant ($p<0.05$) effect of E_2 on GnRH-induced GTH secretion compared to blank.

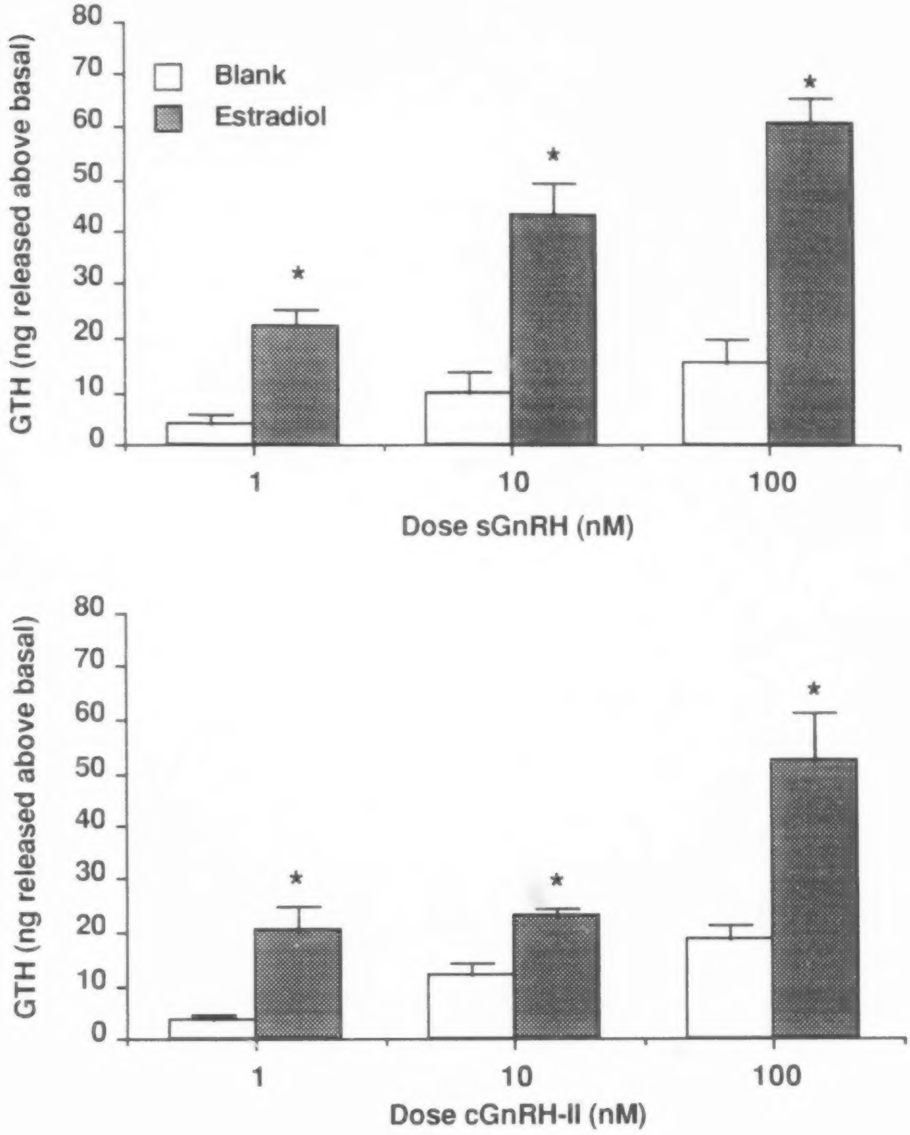

Fig. 3.2. The effects of intraperitoneal implantation of E_2 on the *in vitro* GTH release response to sGnRH (upper panel) and cGnRH-II (lower panel) in sexually recrudescent female goldfish. Data are the mean ± SE of the total ng GTH released above basal (n=4-5 columns). The (*) indicates a significant ($p<0.05$) effect of E_2 on GnRH-induced GTH secretion compared to blank.

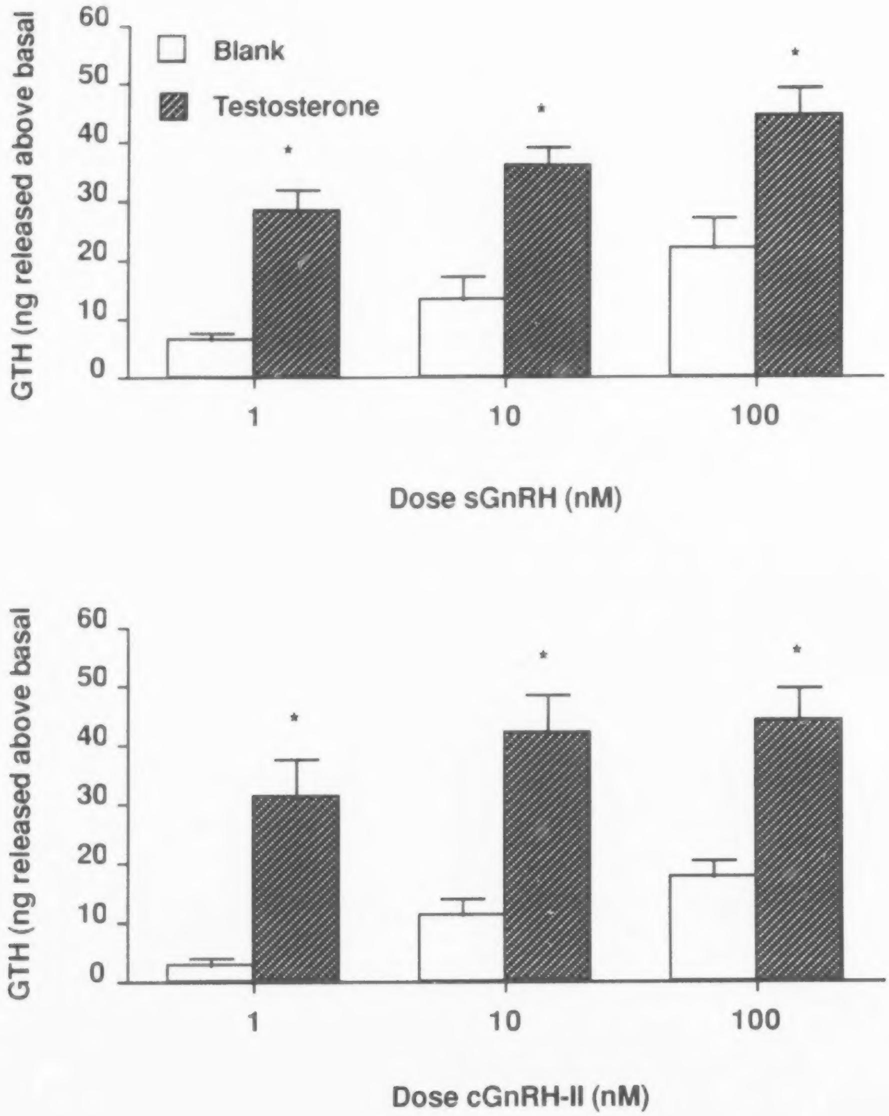

Fig. 3.3. The effects of intraperitoneal implantation of T on the *in vitro* GTH release response to sGnRH (upper panel) and cGnRH-II (lower panel) in sexually recrudescent female goldfish. Data are the mean ± SE of the total ng GTH released above basal (n=4-5 columns). The (*) indicates a significant ($p<0.05$) effect of T on GnRH-induced GTH secretion compared to blank.

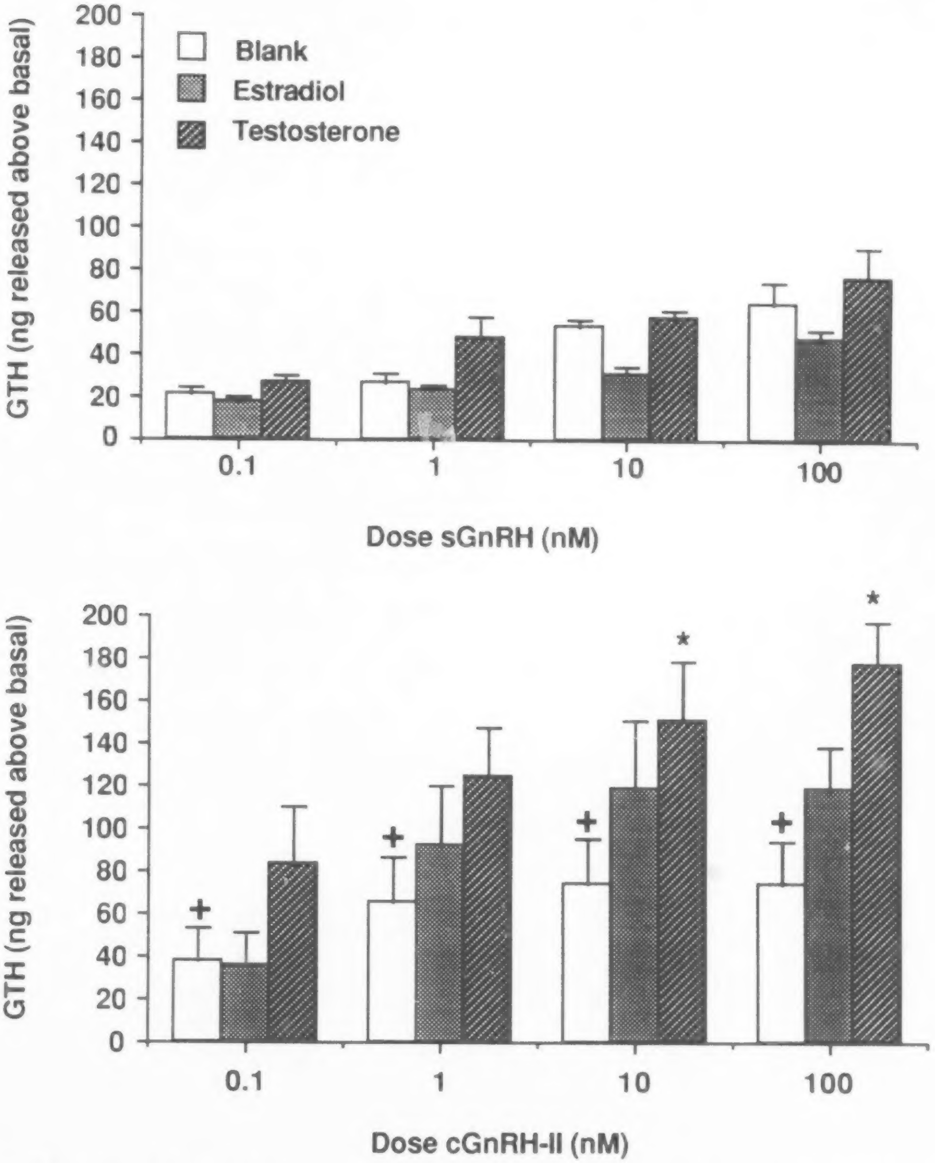

Fig. 3.4. The effects of intraperitoneal implantation of T and E_2 on the *in vitro* GTH release response to sGnRH (upper panel) and cGnRH-II (lower panel) in post-spawning (regressing, atretic ovaries) female goldfish. Data are the mean ± SE of the total ng GTH released above basal. (n=4-5 columns). The (*) indicates a significant ($p<0.05$) effect of T on GnRH-induced GTH secretion compared to blank. The (+) indicates a significantly ($p<0.05$) higher release of GTH with cGnRH-II compared to sGnRH.

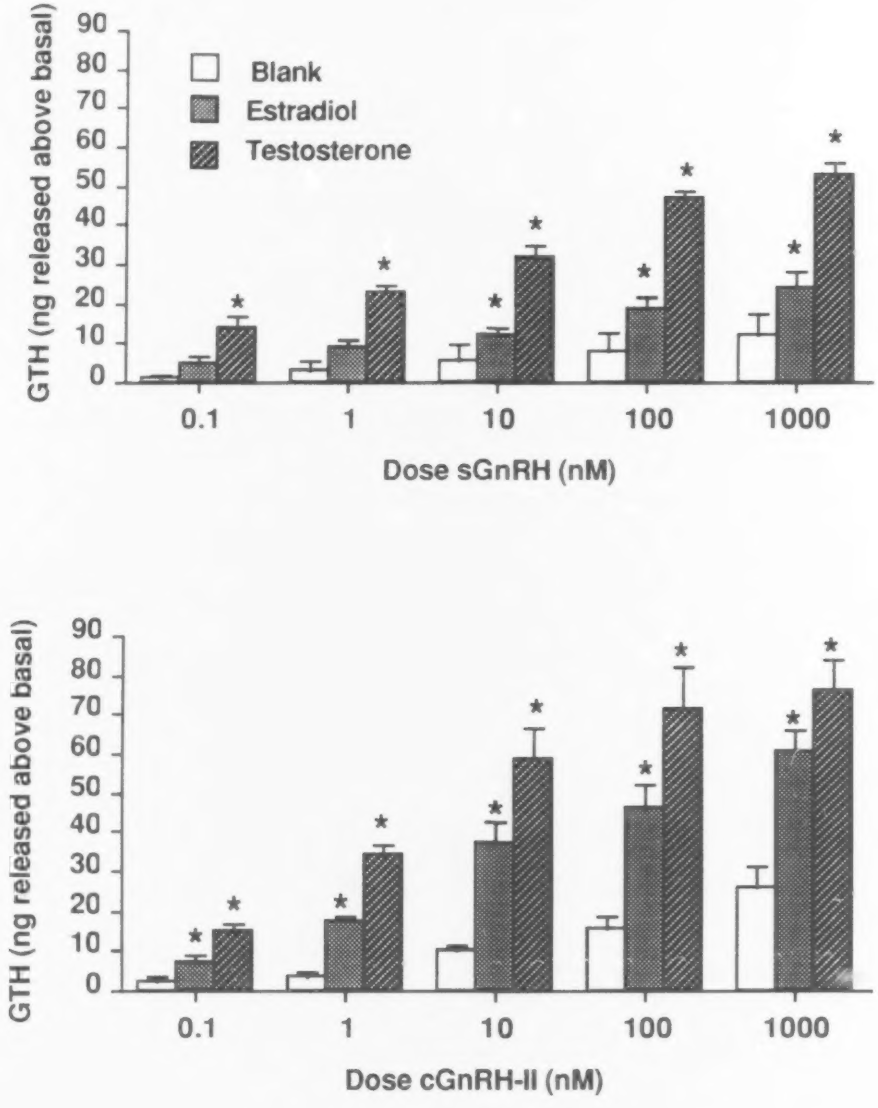

Fig. 3.5. The effects of intraperitoneal implantation of T and E_2 on the *in vitro* GTH release response to sGnRH (upper panel) and cGnRH-II (lower panel) in sexually regressed goldfish. Data are the mean ± SE of the total ng GTH released above basal. (n=5-6 columns). The (*) indicates a significant ($p<0.05$) effect of T and E_2 on GnRH-induced GTH secretion compared to blank.

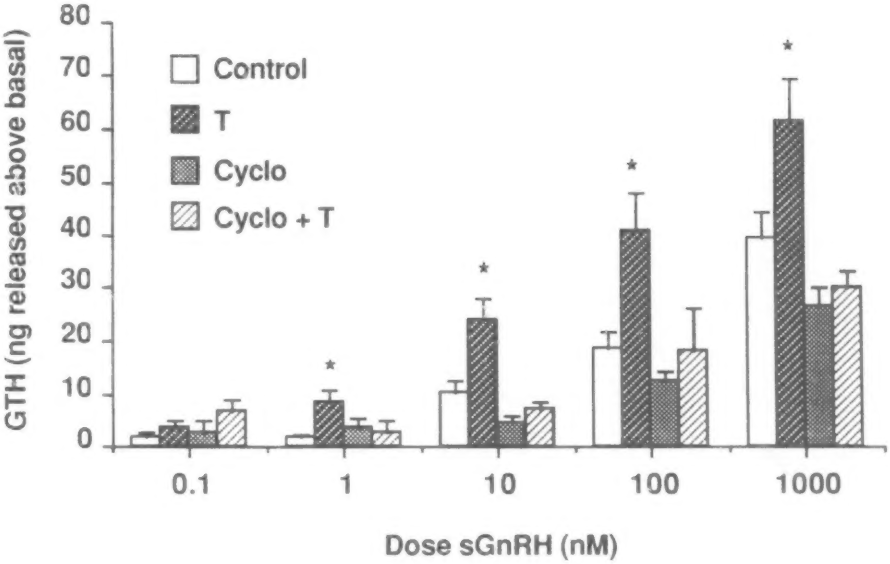

Fig. 3.6. The effects of 24 h *in vitro* exposure to 100 nM T alone or in combination with cycloheximide (25 x 10^{-6} M) on the GTH response of pituitary fragments (sexually regressed fish) to sGnRH. Data are the mean ± SE of the total ng GTH released above basal (n=5-6 columns). The (*) indicates a significant ($p<0.05$) effect of T on sGnRH-induced GTH secretion compared to control.

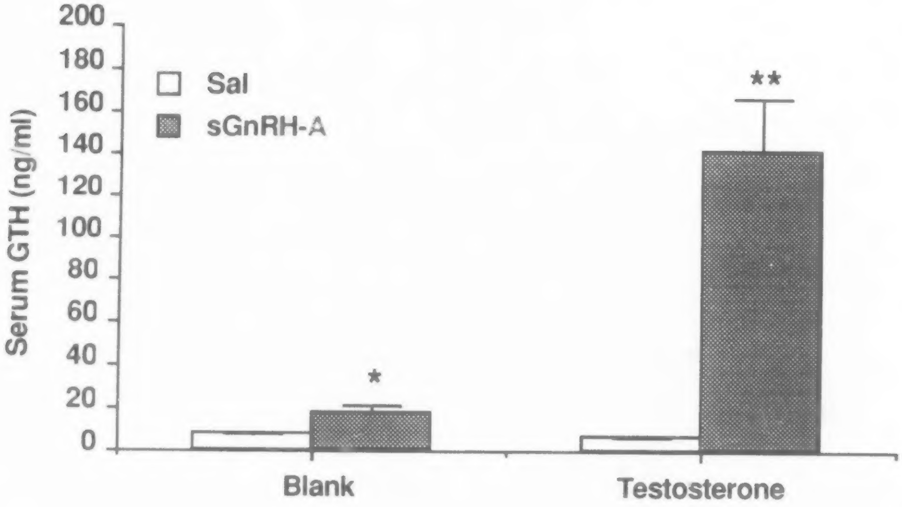

Fig. 3.7. The effects of intraperitoneal implantation of T on the serum GTH response to sGnRH-A (0.01 µg/g; blood collected 6 h later) in sexually regressed goldfish . Data are the mean ± SE (n=12) for serum GTH (ng/ml). The (*) indicates a significant ($p<0.05$) effect of sGnRH-A and the (**) indicates a potentiating ($p<0.05$) effect of T.

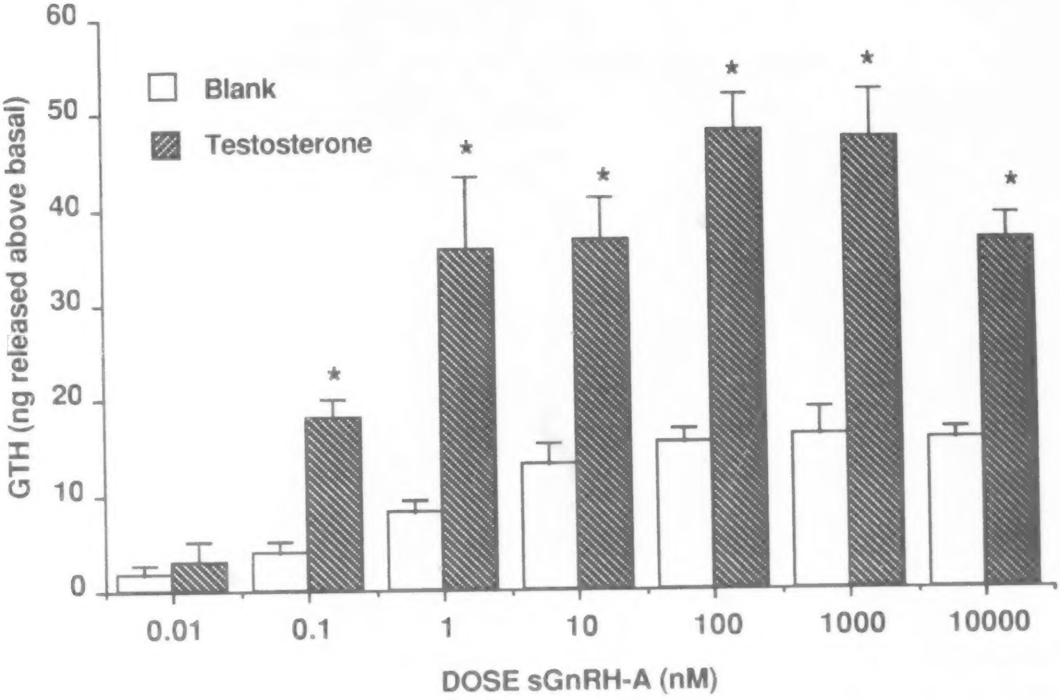

Fig. 3.8. The effects of intraperitoneal implantation of T on the *in vitro* GTH release response to sGnRH-A in July. Data are the mean ± SE (n=8 columns). The (*) indicates a significant ($p<0.05$) effect of T on sGnRH-A induced GTH secretion compared to blank.

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4. INTERACTION OF GONADAL STEROIDS WITH BRAIN CATECHOLAMINES AND GONADOTROPIN-RELEASING HORMONE IN THE CONTROL OF GONADOTROPIN SECRETION IN THE GOLDFISH*

Introduction

Numerous reports on the effects of the catecholamines (CA), particularly dopamine (DA) and norepinephrine (NE), on control of gonadotropin (GTH) secretion in mammals have appeared (see Kalra and Kalra, 1983; Barraclough et al., 1984; Ramirez et al., 1984 for extensive reviews). Despite the overwhelming literature on the role of CAs in control of luteinizing hormone (LH) in the laboratory rat, the role of DA remains unclear. For example, DA has been reported to have stimulatory, inhibitory or no effects on LH secretion (Gallo, 1980; Barraclough et al., 1984; Negro-Vilar et al., 1982; Ramirez et al., 1984). NE can also be stimulatory or inhibitory on LH secretion and its actions are dependent on experimental conditions, i.e., dose and site of application, gonadectomy and sex steroid treatment (Parvizi and Ellendorff, 1982; Kalra and Kalra, 1983; Bergen and Leung, 1986). It is generally accepted, however, that NE has a stimulatory role in the proestrus LH surge in normal rats.

Among the vertebrates, teleost fish are unique in that the adenohypophysis is directly innervated by peptidergic (gonadotropin-releasing hormone; GnRH) and aminergic (DA) neurons (see Ball, 1981; Peter et al., 1990 for review). A major concentration of GnRH perikarya in the ventral preoptic region and a minor group of GnRH perikarya in the latero-basal hypothalamus innervate the proximal pars distalis (PPD; Kah , 1986), where the gonadotroph cells are located (Ball, 1981). Dopaminergic neurons from the anteroventral preoptic region also innervate the PPD (Kah, 1986) whereas DA nerve fibres originating in the posterior hypothalamus innervate the neurointermediate lobe (NIL) of the pituitary (Fryer et al., 1985). In a wide range of teleost fish there is a clear DA-mediated inhibition of spontaneous and GnRH-induced GTH secretion (Peter et al., 1986; 1988 for review). Inhibition of DA synthesis, and DA receptor antagonism by a variety of pharmacological agents clearly enhances GTH secretion in the goldfish; however, the effectiveness of treatments reducing dopaminergic inhibition of GTH varies throughout the reproductive cycle. The increase in serum GTH in response to the DA antagonists pimozide (Sokolowska et al., 1985) and domperidone (Omeljaniuk et al., 1989; Sloley et al., 1991) is greatest in sexually mature goldfish, suggesting that the DA inhibitory tone is

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(*)-A version of this chapter has been accepted for publication. Trudeau, Sloley, Wong and Peter, 1992. General and Comparative Endocrinology: in press.

greatest when gonadal function (i.e. sex steroid secretion) is maximal. NE has been shown to have a slight stimulatory effect on GTH secretion in sexually regressed but not sexually mature goldfish (Chang and Peter, 1984). Although the mechanism of this NE-induced stimulation of GTH secretion is not clear, NE can stimulate GnRH release from preoptic-anterior hypothalamic slices *in vitro* (Yu and Peter, 1991; Yu et al, 1991) and also has a minor effect directly on the gonadotroph cell to stimulate GTH secretion (Chang et al., 1991).

Gonadal steroids have been shown to modulate GTH secretion in a variety of vertebrate species (see Sharp, 1983; Fink, 1988; Karsh, 1987; Pavgi and Licht, 1989). In mammals, the positive and negative effects on GTH secretion are mediated via mechanisms that involve changes in GTH synthesis, pituitary cell responsiveness to GnRH and changes in GnRH receptor number (Kalra and Kalra, 1983; Clayton et al., 1985; Conn et al., 1987). Another possible mechanism by which gonadal steroids may affect GTH secretion is through interaction with CA systems in the brain and/or pituitary. In rats, gonadal steroids act to affect hypothalamic CA turnover and, subsequently, GnRH and GTH secretion (Barraclough et al., 1984; Ramirez et al., 1984). Since T and E_2 enhance (Trudeau et al., 1991) and DA inhibits (Peter et al., 1986) GnRH-induced GTH secretion in goldfish, it is possible that sex steroids act by modulating DA-inhibition of GTH secretion. Although the involvement of brain CA in gonadal steroid feedback control of GTH secretion in teleosts has been suggested by several authors, (i.e., De Leeuw et al., 1987; Timmers and Lambert, 1989; Manickam and Joy, 1990), these studies provided no direct evidence for such interactions. The present report examines the interaction of T and E_2 with CA in the control of GTH secretion.

Materials and Methods

Animals

Common or comet varieties of goldfish (*Carassius auratus*) weighing 30-60 g were purchased from a commercial supplier (Grassyforks Fisheries, Martinsville, Ind, U.S.A.). Prior to experimentation, fish were acclimated to $17^{o}C$ and natural-simulated photoperiods (Edmonton, Alberta). All animals were fed to satiation each morning with trout pellets.

Steroid treatments and blood collection

Solid silastic pellets containing T, E_2 or no steroid (control) were manufactured and implanted intraperitoneally as described (Trudeau et al., 1991). Fish received a steroid dose of 100 µg/g body weight. Blood samples were taken from anaesthetized (tricane methanesulfonate) fish by caudal puncture using 25 G needles. Blood was allowed to clot for 16-24 h (at 4° C), and serum was collected by centrifugation and kept frozen (-20° C) until hormone analysis.

Experimental protocols

Effect of steroid treatment on catecholamine turnover in the brain and pituitary

Catecholamine turnover rates (CA-TOR) were determined following tyrosine hydroxylase inhibition by α-methyl-para-tyrosine methyl ester HCl (MPT; 240 μg/g) by modification of the methods of Brodie et al. (1966) and Rance et al. (1981). Sexually regressed (August) or sexually recrudescent (November) goldfish were implanted with T or E_2 and five days later injected i.p. with MPT. Blood samples were taken and animals were sacrificed by severing the spinal cord immediately after (0 h) and again at 2 h and 4 h following MPT. Brains of fish were rapidly removed through an opening made in the top of the skull and placed on an ice cold glass plate. The hypothalamus (HYP) and telencephalon including preoptic area (TEL-POA) were rapidly removed, placed in preweighed 1.5 mL polypropylene microtubes and immediately frozen on dry ice. The pituitary (PIT) was removed and placed in a 1.5 mL polypropylene microtube and frozen on dry ice. Dissections took less than 2 minutes. Tissues were stored frozen at -28° C until estimation of amine concentrations (usually less than one week).

Concentrations of DA and NE were determined by high performance liquid chromatography (HPLC) with electrochemical detection as described (Sloley and Orikasa, 1988; Sloley et al., 1991). Amines were extracted from tissues by homogenizing tissues, using an ultrasonic tissue disruptor, in 500 μL (HYP, TEL-POA), or 70 μL (PIT) 0.2 N perchloric acid containing 0.1 mM sodium metabisulphite, 0.25 mM EDTA and 0.1 μg/mL isoproterenol as internal standard. Homogenates were centrifuged for 10 minutes at 12,800 X g and 50 μL of the supernatant injected directly onto the HPLC column (C-18 Ultrasphere, Beckman). As single fish pituitaries were difficult to weigh accurately, estimates of pituitary DA concentrations were based on the amount of protein present. Pituitary protein determinations were made using the BIO-RAD protein assay method using bovine serum albumin as standard (Bradford, 1976).

The effects of steroid treatment on the serum GTH response to GnRH or domperidone

At various times of the year, at 5 days following steroid implantation, fish were injected i.p. with either saline (0.6 % NaCl), [D-Arg^6, Pro^{9}NEt]-salmon GnRH (sGnRH-

A; 0.01 µg/g) or the DA receptor antagonist domperidone (DOM; 10 µg/g) for assessement of the in vivo GTH release response. In addition to domperidone's antagonist properties, it has recently been shown to selectively deplete PIT DA in the goldfish (Sloley et al., 1991). The site of DOM action, however, is restricted to the PIT (Omeljaniuk et al., 1987; Sloley et al., 1991) . The serum GTH response was assessed at 6 or 24 h following injection of sGnRH-A or DOM, respectively.

The effects of DA depletion on the potentiating effect of testosterone on GnRH-stimulated GTH release

Since T alters DA-TOR in the brain and PIT (see results section) and also potentiates GnRH-induced GTH secretion (Trudeau et al., 1991) we were interested in determining whether DA was involved in the positive feedback action of T. Goldfish were injected i.p. with saline or MPT (240 µg/g). In the first experiment, sexually recrudescent female fish were injected with saline or sGnRH-A (0.01 µg/g) 5 days after MPT treatment and the serum GTH response assessed 6 h later. At the end of the experiment, saline and MPT-injected animals were sacrificed and brain and pituitaries dissected for determination of DA and NE concentrations. This experiment demonstrated the effectiveness of MPT in long-term inhibition of CA synthesis and resultant depletion of tissue CA concentrations. In a second experiment, sexually recrudescent males were injected with saline or MPT, and implanted with T (100 µg/g) 2 days later. Five days after T treatment, fish were injected with saline or sGnRH-A to assess the serum GTH response. Pituitary DA levels were also measured at the end of the experiment (7 days after MPT injection).

The effects of steroid treatment on dopaminergic inhibition of GnRH-stimulated GTH secretion in vitro

The effects of in vivo T and E_2 implantation on *in vitro* GTH secretion from pars distalis (p.d.) fragments of sexually recrudescent female goldfish were examined using an established perifusion system (Marchant et al., 1989). Following dissection, p.d. fragments were preincubated for 2 hours prior to experimentation. Fragments were exposed at hourly intervals to 2-minute pulses of 100 nM sGnRH in the absence or presence of increasing concentrations (1-1000 nM) of the specific DA type-2 receptor agonist, LY 171555 (generously donated by Dr. M.L. Johnson, Lilly Research Ltd., USA). At each dose, LY 171555 was administered continuously 30 minutes before and 30 minutes following a sGnRH pulse. Medium effluent was collected at 5 minute intervals. The mean GTH levels of the 3 samples (15 minutes) preceeding a sGnRH pulse was considered indicative of basal secretion. The GTH response to sGnRH was quantified as total ng GTH released above basal. An increment of GTH following sGnRH application was included as part of a response if it was greater than 1 S.E.M above basal secretion.

Radioimmunoassay

Gonadotropin (GTH) levels in serum and perifusion medium were measured using a double antibody radioimmunoassay (Peter et al., 1984) specific for GTH-II (Van Der Kraak et al., 1992).

Statistical analysis

Serum GTH levels and initial brain and pituitary CA contents were analyzed by the least squares method of analysis of variance (Statistical Analysis Systems, Inc.); treatment group means were considered statistically different if $p<0.05$. Rate constants (slope of CA decline after MPT injection) and turnover rates (TOR) were calculated according to Brodie et al. (1966) and Rance et al. (1981). Rate constants (K) were calculated by the method of least squares after log transformation of CA concentration, and TOR is the product of K and initial CA concentrations (CA-o). To determine significant differences between turnover rates, Z values were calculated as reported (Rance et al., 1981) and entered on a table of standard normal distribution. Turnover rates were considered statistically different if $p<0.01$. In an initial study, MPT was found to have maximal CA synthesis inhibition by 4 h, therefore, rate constants were calculated based on samplings at 0, 2 and 4 h following MPT injection. The IC_{50} values for *in vitro* LY 171555 inhibition of sGnRH-stimulated GTH release were estimated using the Allfit program (DeLean et al., 1978). Estimates of IC_{50} in this experiment were compared statistically using 95 % confidence intervals.

Results

The effects of steroid treatment on catecholamine turnover in the brain and pituitary of the goldfish

The effects of steroid treatment on CA-TOR inTEL-POA, HYP and PIT in sexually regressed goldfish are shown in Table 4.1. Initial tissue concentrations of NE and DA were not affected by steroid implantation. Following MPT injection, CA levels decreased progressively and at different rates according to treatment group. Norepinephrine TOR in

TEL-POA and HYP was decreased by E_2 but was unaffected by T implantation in sexually regressed fish. Furthermore, in control implanted animals, NE-TOR was higher ($p<0.01$) in TEL-POA than HYP. Dopamine-TOR in TEL-POA and HYP was unaffected by E_2 but was decreased by T implantation. Dopamine-TOR in PIT was increased ($p<0.01$) by both E_2 and T implantation. Norepinephrine was not detected in pituitary tissues. In control implanted animals, DA-TOR was lower ($p<0.01$) in TEL-POA than HYP. Gonadotropin secretion following MPT injection in regressed fish is depicted in Fig. 4.1 (upper panel). Steroid implantation did not affect basal GTH levels and injection with MPT did not affect serum GTH levels in control, T or E_2 treated fish.

The effects of steroid treatment on TEL-POA, HYP and PIT CA-TOR in sexually recrudescent female goldfish are shown in Table 4.2. Initial tissue concentrations of NE and DA were not affected by steroid implantation. Norepinephrine TOR in TEL-POA and HYP was increased by E_2 and decreased in the TEL-POA by T implantation in sexually recrudescent fish. Furthermore, in control implanted animals, NE-TOR was higher ($p<0.01$) in TEL-POA than HYP. Dopamine TOR in TEL-POA was increased by E_2 and T treatment whereas DA-TOR in the HYP was unaffected by either steroid. In control implanted animals, DA-TOR was lower ($p<.01$) in TEL-POA than HYP. In the PIT, E_2 increased DA-TOR only slightly ($p>0.01$) whereas T significantly increased DA-TOR. Gonadotropin secretion following MPT injection in sexually recrudescent fish is depicted in Fig. 4.1 (lower panel). In control implanted animals serum GTH levels at 4 hours after MPT injection were elevated relative to values at 0 h. At 2 hours after initial MPT injection, serum GTH levels were significantly ($p<0.05$) increased in both T and E_2 implanted animals compared to control GTH levels. By 4 hours, serum GTH levels were similar in all groups.

Brain serotonin levels were also slightly decreased by MPT injection (data not shown), suggesting that MPT action is not exclusively on tyrosine hydroxylase but also involves inhibition of tryptophan hydroxylase, as has been shown in rats (Gopalan et al., 1989).

The effects of steroid treatment on the serum GTH response to sGnRH-A or the DA-receptor antagonist domperidone

The serum GTH responses to sGnRH-A in T- and E_2-implanted sexually regressed fish are shown in Fig. 4.2. Unstimulated GTH levels were not affected by steroid treatments. Control animals did not respond to sGnRH-A alone, but implantation with either E_2 or T enhanced the GTH response to sGnRH-A; T was particularly effective in this regard. The effects of steroid treatment on the serum GTH response to DOM are shown in Fig. 4.3. In sexually regressed control-implanted female fish (Fig. 4.3; upper panel), DOM injection resulted in a small but significant elevation of serum GTH. Implantation of T and E_2 resulted in enhanced GTH responses to DOM. In female fish in late ovarian recrudescence (Figure 4.3; lower panel), the GTH response to DOM was greater than that in sexually regressed fish ($p<0.05$). Implantation of T but not E_2 resulted in an enhanced GTH response to DOM.

The effect of DA depletion on the potentiating effect of testosterone on GnRH-stimulated GTH release

The effects of MPT on brain and pituitary CA levels in sexually recrudescent female goldfish are shown in Table 4.3. DA levels in TEL-POA, HYP and PIT were severely depleted five days following a single MPT injection. Similarly, NE levels in the TEL-POA and HYP were also significantly lower in MPT-injected fish. Injection of 0.01 µg/g sGnRH-A caused a small but significant ($p<0.05$) stimulation of GTH release in controls and the GTH response to sGnRH-A was clearly potentiated by MPT pretreatment (Figure 4.4). In a second experiment with sexually recrudescent males, sGnRH-A stimulated GTH release, and T implantation enhanced this response (Fig. 4.5). Again, injection of MPT also potentiated the GTH response to sGnRH-A. When males were treated both with T and MPT, the GTH response was not different from that in T-implanted or MPT-injected males. Pituitary DA levels in MPT injected fish were depleted as in the previous experiment and were 0.83 ± 0.15 and, 2.43 ± 0.28 pg/µg protein for MPT-injected and controls, respectively ($p<0.05$).

The effects of steroid treatment on dopaminergic inhibition of GnRH-stimulated GTH secretion in vitro

The effects of *in vivo* implantation of T and E_2 on the *in vitro* secretion of GTH from p.d. fragments are shown in Fig. 4.6. The first pulse of 100 nM sGnRH, in the absense of LY171555, stimulated GTH release for p.d. fragments in blank implanted animals and the GTH response was enhanced ($p<0.05$) in p.d. fragments from E_2 and T-implanted fish. Application of increasing doses of the specific DA type-2 receptor agonist LY 171555 progressively inhibited the response to 100 nM sGnRH in all experimental groups. The IC_{50} estimates for the inhibitory effects of LY 171555 on sGnRH-stimulated GTH secretion were not different ($p>0.05$) between groups, and were 0.9 ± 0.3 nM, 1.2 ± 0.4 nM and 1.4 ± 0.1 nM for blank, E_2 and T, respectively.

Discussion

The present study demonstrates that sex steroids increase pituitary responsiveness to GnRH and also alter catecholaminergic neuronal activity in a seasonally breeding teleost fish, the goldfish. Amine turnover estimates were obtained using a non-steady state method (Rance et al., 1981) and steroid implantation affected CA-TOR in a tissue specific manner. CA-TOR estimates following MPT injection are generally considered specific (Barraclough et al.,1984; Ramirez et al.,1984); however, interpretation of the turnover data can be confounded by the observation that brain serotonin concentrations were slightly reduced by MPT. Treatment with either T or E_2 in sexually regressed or sexually recrudescent goldfish did not affect initial concentrations of NE or DA. This is in contrast to observations in catfish (*Clarias batrachus*) where E_2 has been shown to affect hypothalamic CA levels (Manickam and Joy, 1990). A possible explanation of this difference may be that the mode and duration of E_2 administration was different in the two studies; Manickam and Joy (1990) injected ovariectomized catfish daily for 3 days with E_2 whereas we implanted gonad-intact goldfish with solid silastic pellets containing T and E_2 to achieve circulating sex steroid levels (Trudeau et al., 1991) similar to those at ovulation in goldfish (Kobayashi et al., 1986). Implantation of E_2 decreased NE-TOR in TEL-POA and HYP whereas T was without effect in regressed goldfish. In contrast, implantation of T but not E_2 decreased DA-TOR in TEL-POA and HYP. Sex steroids were also found to affect CA-TOR in sexually recrudescent animals, but neuronal responses were different than those seen in regressed goldfish. In recrudescent fish, E_2 increased and T decreased NE-TOR in the TEL-POA. In the HYP, E_2 increased NE-TOR whereas T was without effect. From these results it seems that E_2 is more effective than T in altering brain NE neuronal activity, but whether the response is to decrease or increase NE-TOR seems to be dependent on sexual maturity and on the tissue examined. The response of DA neurons to sex steroids was also affected by sexual stage. In regressed fish, T clearly decreased DA-TOR in TEL-POA and HYP, but E_2 was without effect. In recrudescent animals, both T and E_2 increased DA-TOR in the TEL-POA, but DA-TOR in the HYP was unaffected by steroid treatment. In the PIT both steroids increased DA-TOR; in regressed animals E_2 was more effective than T, whereas in recrudescent animals only T was effective.

At present it is difficult to relate steroid-induced changes in CA turnover with subsequent changes in GTH secretion. In both sexually regressed and recrudescent goldfish, gonadal steroids enhance GTH secretion by potentiating the GTH response to GnRH without affecting basal GTH secretion (Trudeau et al., 1991; present results), and at the same time gonadal steroids also increase DA-TOR in the PIT. Since DA is a potent inhibitor of GTH release (Peter et al., 1986), GnRH release (Yu et al., 1991) and GnRH receptor capacity (DeLeeuw et al., 1989), increased PIT DA-TOR may act to reduce the stimulatory action of endogenous GnRH, thus preventing GTH release and maintaining low basal GTH secretion in spite of enhanced PIT responsiveness to GnRH. That increased PIT DA-TOR following steroid treatment represents a functional increase in inhibitory tone is supported by the observation that gonadal steroids increase the GTH response to DOM. A role for DA in mediating sex steroid negative feedback has also been suggested in some mammalian species (Meyer and Goodman, 1985; Docke et al., 1987). DA synthesis inhibition by MPT (present study) or DA receptor antagonism by DOM (Omeljaniuk et al., 1989; Sloley et al., 1991) results in secretion of GTH in goldfish with developing but not regressed gonads, further indicating a positive relationship between endogenous gonadal steroids and modulation of inhibitory dopaminergic inputs to the gonadotroph cell. Additionally, in control implanted goldfish PIT DA-TOR increases from August (sexually regressed fish) to November (sexually recrudescent fish), when circulating sex steroid levels are also increasing (Kobayashi et al.,1986). Further evidence for a functional link between DA turnover and control of GTH secretion has been presented by Dulka et al. (1991). These researchers have demonstrated that DA-TOR decreases during the surge in GTH secretion induced by exposure of male goldfish to the sex pheromone 17α, 20β-dihydroxyprogesterone.

Steroid-induced changes in TEL-POA and HYP CA neuronal activity could act to regulate brain GnRH. Dopaminergic perikarya and fibres have been localized in the preoptic area (Kah, 1986; Hornby et al., 1987) in proximity to GnRH perikarya (Kah, 1986) in the goldfish. DA is a potent inhibitor of GnRH release from goldfish preoptic-anterior hypothalamic slices and PIT fragments *in vitro* (Yu and Peter, 1991; Yu et al.,1991) and pimozide, a DA-receptor antagonist that crosses the blood-brain barrier, increases TEL-POA and PIT GnRH levels in goldfish (Yu and Peter, 1990). Therefore, any alterations in TEL-POA and PIT DA-TOR could conceivably act to regulate the activity of GnRH perikarya and terminals, respectively. In the case of regressed fish, decreased TEL-POA DA-TOR in response to T could be indicative of a reduction in DA inhibition of GnRH release and this could be part of a mechanism triggering onset of gonadal recrudescence. Interestingly, there is a temporary increase in plasma levels of sex steroids at the onset of gonadal recrudescence in goldfish (Kobayashi et al., 1986) which may have important feedback actions on the brain-pituitary axis. In recrudescent goldfish, increased TEL-POA DA-TOR in response to T and E_2 could be indicative of increased DA inhibition of GnRH neuronal activity, serving to help keep the brain-pituitary system in check and prevent excessive GTH secretion. Noradrenergic perikarya and fibres have been localized in the preoptic area (Hornby and Piekut, 1990) and NE stimulates GnRH release from goldfish preoptic-anterior hypothalamic slices *in vitro* (Yu and Peter, 1991; Yu et al., 1991) and also stimulates GTH release *in vivo* in sexually inactive goldfish (Chang and Peter, 1984). Steroidal modulation of noradrenergic activity could also act to affect GnRH neuronal function (Condon et al., 1986). In the goldfish, as in the laboratory rat (Kalra and Kalra, 1983; Ramirez et al., 1984), it may be that gonadal steroids modulate GnRH and GTH release via both positive and negative actions on CA neurons. In goldfish, sex steroids were found to affect HYP CA-TOR, but it is unclear if this is related to control of GTH secretion; dopaminergic perikarya located in the posterior HYP innervate the NIL and not the PPD. Therefore, changes in HYP DA-TOR induced by T may be related to control of NIL and not PPD function. However, a role for HYP CA in control of GTH secretion cannot be ruled out since a minor group of GnRH perikarya have been localized in the latero-basal HYP (Kah, 1986). Although the HYP is densely innervated by noradrenergic neurons (Hornby and Piekut, 1990) their role in control of PIT function is unknown. Nevertheless, E_2 modulates NE-TOR in the HYP of the goldfish. We also tested whether changes in CA neuronal function was a prerequisite for the positive action of T on GnRH-induced GTH secretion. Testosterone-induced alterations in brain CA-TOR is dissociable from the positive action of T on pituitary responsiveness, since the potentiating effect of T implantation was not affected by severe depletion of brain and pituitary CA levels by MPT pretreatment.

It is not known how sex steroids affect catecholaminergic neuronal function, which represents a limitation to interpreting their effects on catecholamine turnover. Estimates of DA or NE-TOR using the non-steady state method described likely represents a composite change in CA release, degradation and/or reuptake. Testosterone and/or E_2 may influence catecholamine turnover by affecting the activity of enzymes involved in DA and NE synthesis (Kizer et al., 1974) or degradation (Manickam and Joy, 1989). It is also possible that gonadal steroids could affect DA-mediated inhibitory action by influencing DA binding sites in the goldfish pituitary (Omeljaniuk and Peter, 1989), since steroids have been shown to alter DA receptor number in the pituitary of rats (Bression et al., 1985; Pilotte et al., 1984). However, the main effect of T and E_2 seems to be alteration of PIT DA-TOR and not gonadotroph sensitivity to DA. Inhibition of sGnRH-induced GTH secretion by LY 171555, a specific DA type-2 receptor agonist, was not affected by steroid pretreatment, suggesting that DA receptor function is not changed. Sex steroids could also act indirectly, via other neurones (i.e. opioid, GABAergic, serotoninergic) and affect catecholaminergic activity (Reiderer et al., 1989).

Several observations suggest that basal and GnRH-stimulated GTH secretion in teleosts are differentially regulated. Testosterone and E_2 do not affect basal GTH levels in the circulation, but both potentiate GnRH-induced GTH secretion in gonad-intact goldfish (Trudeau et al., 1991; present study). In contrast, gonadectomy and subsequent treatments with T or E_2 in female goldfish (Kobayashi and Stacey, 1990) increases and decreases basal GTH secretion, respectively. Although highly speculative, together these results suggest that in goldfish, gonadal steroids have predominantly negative effects on basal GTH secretion, whereas they exert predominantly positive effects on GnRH-stimulated GTH release.

In summary, although exogenous sex steroids do not affect basal GTH secretion in gonad-intact goldfish *in vivo*, they clearly enhance GnRH-induced GTH secretion as well as the GTH responses to the DA-receptor antagonist DOM and to DA synthesis inhibition by MPT. Gonadal steroids increased DA-TOR in the PIT where DA terminals are located. Increased DA-TOR in the PIT does not appear to be associated with an increased gonadotroph sensitivity to DA inhibition of GnRH-stimulated GTH secretion since since IC_{50} estimates for the specific DA type 2 receptor agonist LY 171555 were not affected by steroid treatments. Testosterone and E_2 both alter DA and NE-TOR in the goldfish brain but the significance of this is unknown. Together these results suggest that gonadal steroids in the goldfish have multiple sites of action in the control of GTH secretion. We suggest that following steroid treatment in gonad-intact fish, basal GTH secretion is maintained by predominantly negative neural signals (i.e., increased PIT DA-TOR) and steroids concurrently prime the PIT and enhance the stimulatory effect of GnRH. Gonadal steroids may act to modulate the relative contribution of stimulatory (i.e. GnRH and/or NE) and inhibitory (DA) neuroendocrine factors which ultimately determines the pattern of GTH secretion.

Table. 4.1. The effects of estradiol and testosterone on norepinephrine (NE) and dopamine (DA) turnover rates (TOR) in the telencephalon-preoptic area (TEL-POA), hypothalamus (HYP) and pituitary (PIT) of sexually regressed goldfish (August). Data are presented as mean ± SEM. Calculation procedures are described in Materials and Methods.

	Blank	**Estradiol**	**Testosterone**
TEL-POA NE			
CA-o (ng/g)	1073 ± 44	934 ± 52	1026 ± 58
K	.102 ± .021	.049 ± .027	.079 ± .031
TOR(ng/g/h)	109.3 ± 16.5^{a}	46.6 ± 17.7^{b}	81.3 ± 22.7^{a}
TEL-POA DA			
CA-o (ng/g)	140 ± 6	129 ± 6	138 ± 7
K	.114± .021	.103± .028	.054 ± .023
TOR (ng/g/h)	16.0 ± 2.2^{a}	13.3 ± 2.7^{a}	7.5 ± 2.3^{b}
HYP NE			
CA-o (ng/g)	706 ± 33	671 ± 37	762 ± 44
K	.075 ± .022	.029 ± .025	.084 ± .024
TOR (ng/g/h)	52.9 ± 11.7^{a}	19.7 ± 12.3^{b}	63.8 ± 13.4^{a}
HYP DA			
CA-o (ng/g)	425 ± 24	404 ± 16	422 ± 17
K	.089 ± .022	.083 ± .022	.036 ± .021
TOR (ng/g/h)	37.8 ± 7.0^{a}	33.7 ± 6.5^{a}	15.4 ± 6.8^{b}
PIT DA			
CA-o (pg/μg protein)	1.63 ± .09	1.88 ± .13	1.81 ± .13
K	.086 ± .025	.146 ± .032	.115 ± .026
TOR (pg/μg/h)	.140 ± .030^{c}	.275 ± .016^{a}	.208 ± .008^{b}

CA-o = Catecholamine concentration before injection of MPT (n=15-22).
K = slope of CA depletion following injection of MPT (n=10-15 for 2 and 4 h).
TOR = CA turnover rate (CA-o x K).
a,b,c = TOR with different superscripts are statistically different ($p<0.01$).

Table. 4.2. The effects of estradiol and testosterone on norepinephrine (NE) and dopamine (DA) turnover rates (TOR) in the telencephalon-preoptic area (TEL-POA), hypothalamus (HYP) and pituitary (PIT) of female goldfish in early stages of gonadal recrudescence (November). Data are presented as mean ± SEM. Calculation procedures are described in Materials and Methods.

	Blank	**Estradiol**	**Testosterone**
TEL-POA NE			
CA-o (ng/g)	1186 ± 83	1337 ± 51	1168 ± 73
K	.067 ± .023	.081 ± .021	.031 ± .027
TOR(ng/g/h)	79.2 ± 22.8^{b}	108.3 ± 24.1^{a}	36.3 ± 26.7^{c}
TEL-POA DA			
CA-o (ng/g)	171 ± 18	211 ± 14	228 ± 48
K	.050 ± .030	.174 ± .031	.149 ± .031
TOR (ng/g/h)	8.6 ± 4.3^{b}	36.7 ± 6.0^{a}	34.0 ± 6.2^{a}
HYP NE			
CA-o (ng/g)	574 ± 42	650 ± 47	511 ± 78
K	.002 ± .025	.051 ± .030	.005 ± .019
TOR (ng/g/h)	1.0 ± 9.8^{b}	33.1 ± 14.7^{a}	2.4 ± 8.2^{ab}
HYP DA			
CA-o (ng/g)	451 ± 17	483 ± 43	408 ± 83
K	.056 ± .021	.050 ± .032	.040 ± .026
TOR (ng/g/h)	25.4 ± 7.6^{a}	24.3 ± 13.3^{a}	16.4 ± 8.8^{a}
PIT DA			
CA-o (pg/µg protein)	2.21 ± .27	2.40 ± .45	2.64 ± .65
K	.141 ± .053	.167 ± .047	.306 ± .065
TOR (pg/µg/h)	.313 ± .173^{b}	.401 ± .201^{ab}	.809 ± .320^{a}

CA-o = Catecholamine concentration before injection of MPT (n=9-13).
K = slope of CA depletion following injection of MPT (n=9-13 for 2 and 4 h).
TOR = CA turnover rate (CA-o x K).
a,b,c = TOR with different superscripts are statistically different ($p<0.01$).

Table 4.3. Brain and pituitary catecholamine levels in female goldfish 5 days after MPT (240 µg/g) injection. Data are mean ± SEM (n=7).

	Catecholamine concentration	
TEL-POA	**DA (ng/g)**	**NE (ng/g)**
control	151±16	1409±147
MPT	52± 2*	786±43*
HYP	**DA (ng/g)**	**NE (ng/g)**
control	566±13	1097±73
MPT	221±5*	590±31*
PIT	**DA (pg/µg protein)**	
control	5.02±0.54	
MPT	1.53±0.15*	

(*) significantly different from control, $p<0.05$.

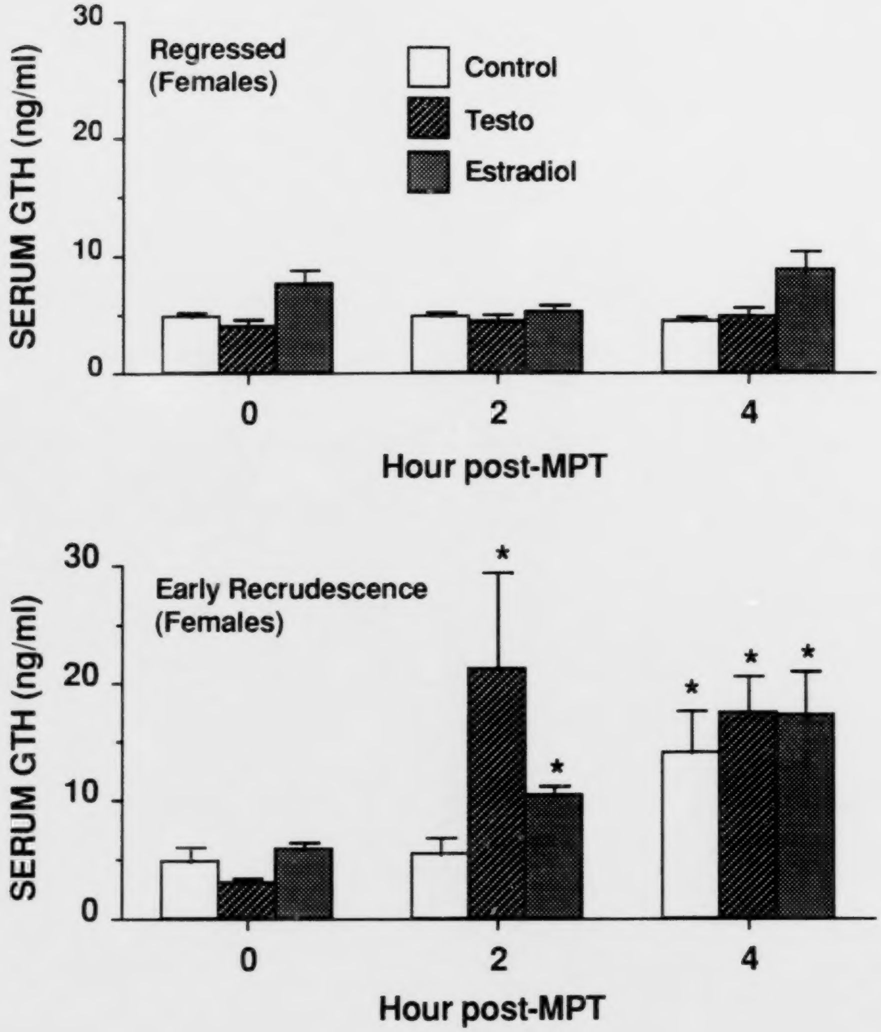

Fig. 4.1. The effects of intraperitoneal implantation of T and E_2 on the serum GTH response to MPT (240 µg/g) in sexually regressed (upper panel; n= 9-12) and sexually recrudescent (lower panel; n=15-22) female fish. Sexually regressed fish (August, GSI=1 %) did not respond to MPT. For sexually recrudescent fish (November, GSI= 2.5 %) the (*) indicates an elevation ($p<0.05$) of GTH relative to 0 h. Data are presented as mean ± SEM.

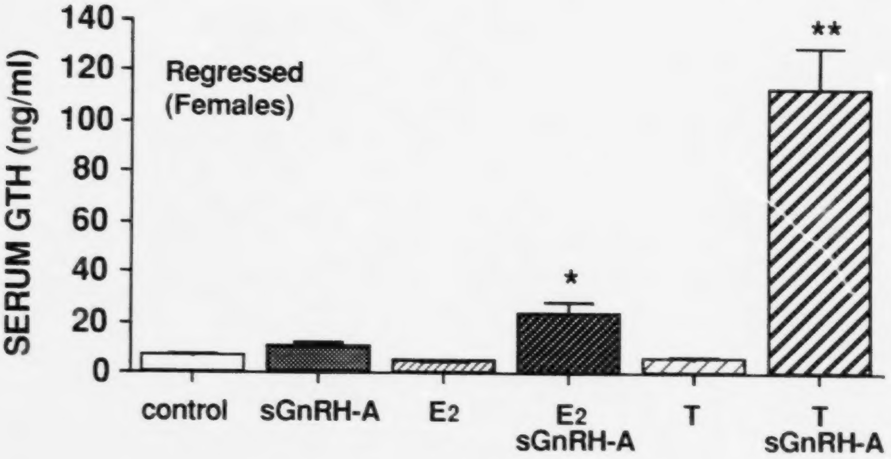

Fig. 4.2. The effects of intraperitoneal implantation of T and E_2 on the serum GTH response to sGnRH-A (0.01 µg/g) in sexually regressed female goldfish (GSI=1 %). Injection of sGnRH-A alone did not affect ($p>0.05$) serum GTH levels. The potentiating effect ($p<0.05$) of steroid implantation on sGnRH-A induced GTH secretion is indicated by (*). Data are presented as mean ± SEM; n=11-13.

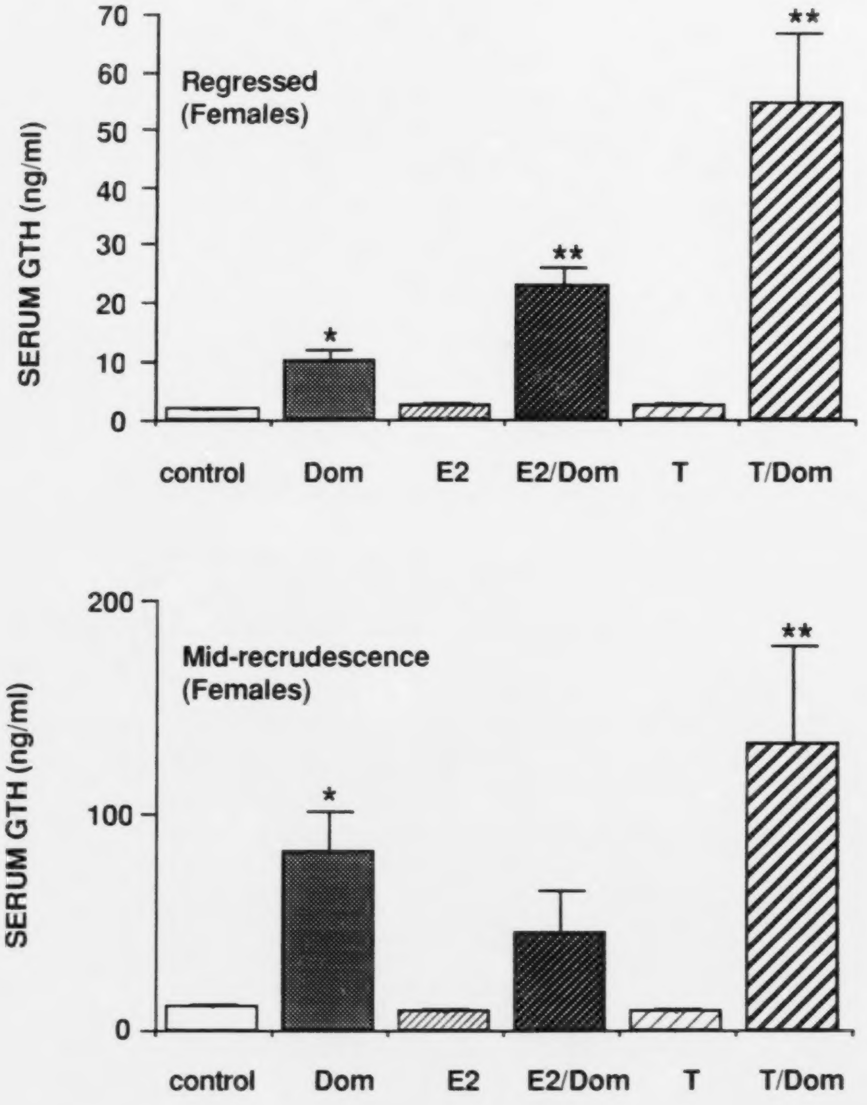

Fig. 4.3. The effects of intraperitoneal implantation of T and E_2 on the serum GTH response to DOM (10 µg/g) in sexually regressed (upper panel; October, GSI=1.4%) and sexually recrudescent (lower panel; February, GSI=6%) female goldfish. The (*) indicates an effect ($p<0.05$) of DOM in blank implanted controls whereas (**) indicates a potentiation of the DOM effect ($p<0.05$) by steroid implantation. Data are presented as mean ± SEM; n=6-10.

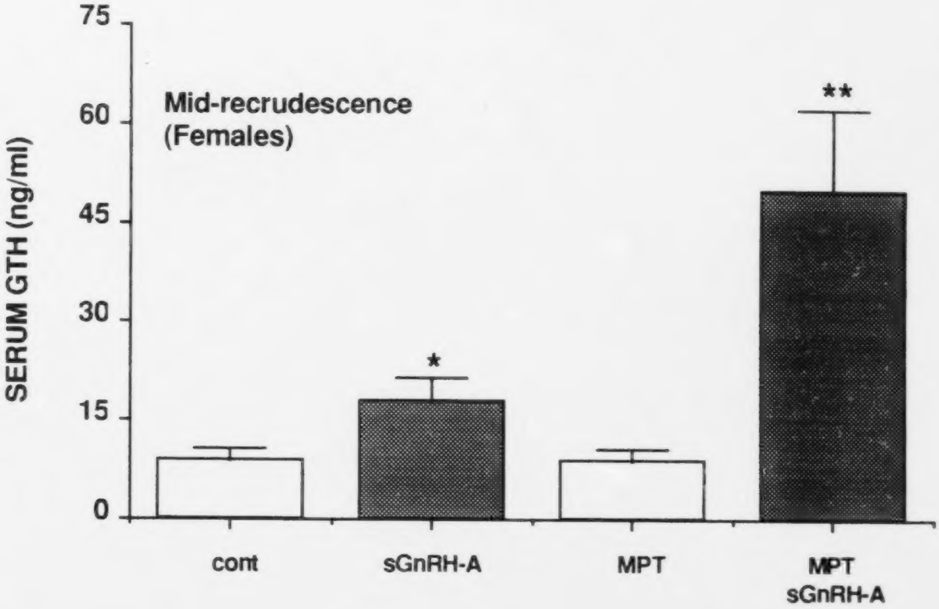

Fig. 4.4. The effects of intraperitoneal injection of MPT (240 μg/g) on the serum GTH response to sGnRH-A (0.01 μg/g) in sexually recrudescent female goldfish (January, GSI= 3%). The (*) indicates a stimulatory effect of sGnRH-A on serum GTH in controls ($p<0.05$) whereas the potentiating effect ($p<0.05$) of MPT on sGnRH-A induced GTH secretion is indicated by (**). Data are presented as mean ± SEM; n= 12.

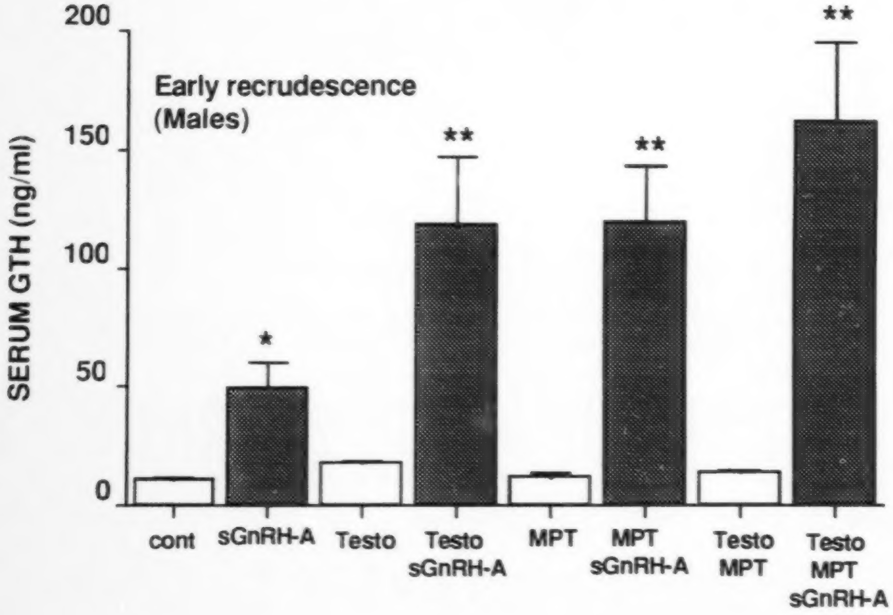

Fig. 4.5. The effects of pretreatment with MPT (240 μg/g) on the serum GTH response to sGnRH-A (0.01 μg/g) in T-implanted male goldfish in early stages of gonadal recrudescence (February, GSI= 1%). Injection of sGnRH-A alone stimulated ($p<0.05$) GTH secretion. The potentiating effect ($p<0.05$) of MPT or T implantation on sGnRH-A induced GTH secretion is indicated by (**). Pretreatment with MPT did not further enhance the positive effect of T on sGnRH-A stimulated GTH secretion. Data are presented as mean ± SEM; n=12.

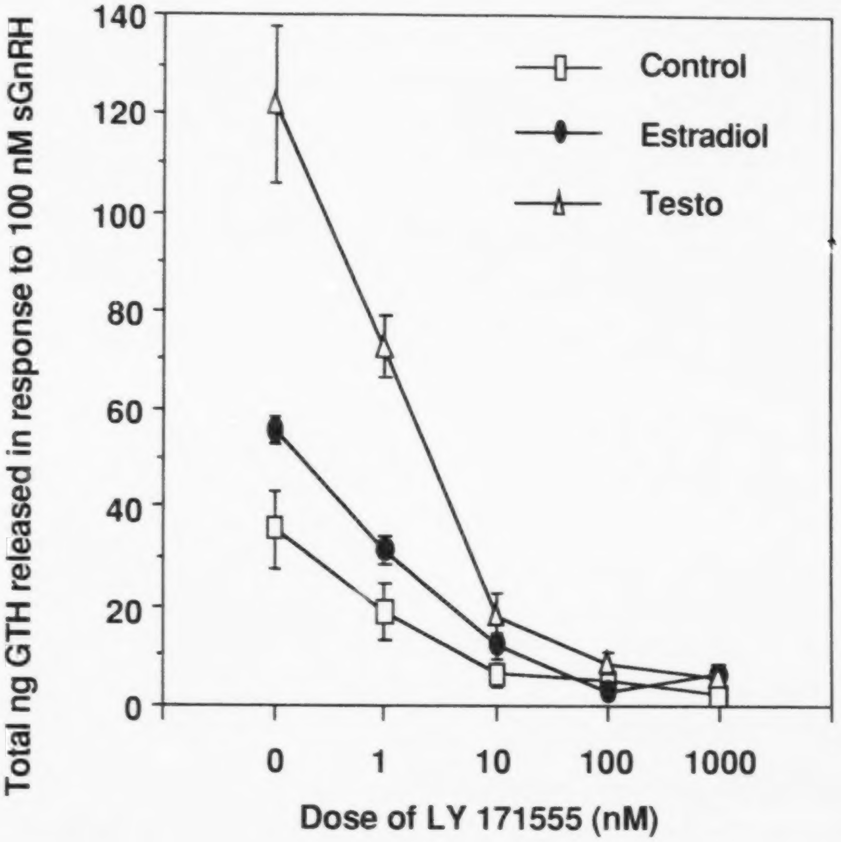

Fig. 4.6. The effects of intraperitoneal implantation of T and E_2 on the *in vitro* GTH response to 100 nM sGnRH in the absence (0 nM) or presence of increasing doses of LY 171555 (1-1000 nM), a DA-type 2 specific agonist, in sexually recrudescent female goldfish (January, GSI= 2.2 %). IC_{50} estimates for LY 171555 in blank, E_2 and T groups were not different ($p>0.05$) and were 0.9 ±0.3 nM, 1.2 ± 0.4 nM and 1.4 ± 0.1 nM, respectively. Data are presented as mean ± SEM; n=4-5 perifusion columns. Basal GTH level over the first 30 minutes was 6-20 ng/ml and was similar for all treatment groups.

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5. MODULATION BY SEX STEROIDS OF γ-AMINOBUTYRIC ACID AND TAURINE, BUT NOT N-METHYL-D,L-ASPARTATE STIMULATION OF GONADOTROPIN SECRETION IN THE GOLDFISH

Introduction

Gamma aminobutyric acid (GABA), glutamate (GLU) and taurine (TAU) are amino acids considered to have important neurotransmitter or neuromodulator functions in vertebrates (Guidotti, 1978; McGeer and McGeer, 1989; Huxtable, 1989). Best studied are GABA and GLU, which have also been implicated in the control of luteinizing hormone (LH) secretion in rodents and primates (Price et al., 1978; Tappaz et al., 1982; Gay and Plant, 1987; McCann and Rettori, 1988). In contrast, the effects of TAU on gonadotropin (GTH) secretion are virtually unexplored. Price et al., (1978) have shown that in male rats TAU can inhibit LH release stimulated by N-methyl-D,L,-aspartate (NMA), but TAU had no effects on its own.

Among the vertebrates, teleost fish are unique in that the adenohypophysis is directly innervated by peptidergic (i.e. gonadotropin-releasing hormone; GnRH) and aminergic (i.e. dopamine; DA) neurons originating from the ventral preoptic region (for review see Ball, 1981; Kah, 1986, Peter et al., 1990b); in effect, the median eminence has moved into the adenohypophysis. Salmon gonadotropin-releasing hormone (sGnRH) and chicken GnRH-II (cGnRH-II), the endogenous GnRH peptides in goldfish brain and pituitary (Yu et al., 1988), stimulate gonadotropin (GTH) release (Peter et al., 1990) from the proximal pars distalis where the gonadotroph cells are located (Ball, 1981). Dopamine directly inhibits GTH secretion by activation of DA type 2-like receptors (Peter et al., 1986; Chang et al., 1990). Dopamine also inhibits GnRH release at both the pituitary and preoptic-anterior hypothalamic levels (Yu and Peter, 1991; Yu et al., 1991). Other factors known to stimulate GTH release in goldfish include norepinephrine (Chang et al. 1983; 1991), serotonin (Somoza et al., 1988; Somoza and Peter, 1991) and neuropeptide Y (Peng et al., 1990).

In contrast to neuropeptide and aminergic regulation of GTH secretion in teleosts, little information concerning the involvement of amino acid neurotransmitters exists. In goldfish, GABA is present in the brain and pituitary (Nilsson, 1990; Kah et al., 1992; Sloley et al., 1992), and neurons presumably originating in the nucleus lateralis tuberis (NLT; teleost homologue of mammalian arcuate nucleus) directly innervate the pars distalis (Kah et al., 1987; Martinoli et al., 1990). Glutamate and TAU are also present in brain and pituitary of fish (Nilsson, 1990; Sloley et al., 1992) but their cellular localization has not been determined. We have recently demonstrated that intraperitoneal (i.p.) or brain intracerebroventricular (i.c.) administration of GABA and TAU stimulate acute GTH release in goldfish (Kah et al., 1992; Sloley et al., 1992) but there is no information on the physiological regulation of amino acid neurotransmitter-mediated GTH secretion. Glutamate is also implicated in the control of GTH secretion in goldfish since monosodium glutamate (MSG)-induced lesions of the NLT is associated with GTH release (Peter et al., 1980; Kah et al., 1983) and potentiation of the GTH response to exogenous GnRH (Sloley et al., 1992). The present results further characterize the stimulatory actions of GABA and TAU on GTH release, and demonstrate that NMA-sensitive mechanisms may function in the regulation of GTH secretion in goldfish. In addition, the GTH-releasing activity of these neurotransmitter/neuromodulator substances may be modulated by DA and gonadal steroids.

Materials and Methods

Animals

Common or comet varieties of goldfish (Carassius auratus) weighing 15-40 g were purchased throughout the year from commercial suppliers (GrassyForks Fisheries, Martinsville, IN or Ozark Fisheries, Stoutland, MO, U.S.A.). Fish were acclimated to 17^{o}C, fed and maintained as previously reported (Trudeau et al.,1991).

Experimental Protocols

The effects of GABA and γ– vinyl GABA on GTH release

At various times of the year, the effects of GABA on GTH release *in vivo* were tested in control, gonad-intact and steroid-implanted goldfish. Fish were implanted for various periods, with blank (no steroid), testosterone (T)-, estradiol (E_2)- or progesterone (P_4)-containing solid silastic pellets (100 µg/g BW steroid) as previously described (Trudeau et al., 1991). On the day of experimentation, GABA (100 µg/g; 10 µl/g BW) was dissolved in 0.6 % NaCl and injected intraperitoneally (i.p.). At 30 min following injection blood samples were taken from tricaine methane sulfonate-anaesthetized fish by caudal puncture using 25 G needles.

The effects of the GABA transaminase inhibitor γ- vinyl GABA (GVG; generously donated by Merrell Dow Research Institute, Strasbourg, France) on brain and pituitary GABA and, serum GTH levels were also tested in control and steroid implanted animals. GVG (300 µg/g; 5 µl/g BW) was injected i.p. and blood was collected 2-48 h later. At the termination of blood collection, brains of fish were rapidly removed and placed on an ice cold glass plate. The telencephalon including preoptic area (TEL-POA), hypothalamus

(HYP) and pituitary (PIT) were rapidly removed and frozen on dry ice , and concentrations of GABA estimated by high performance liquid chromatography (HPLC) with fluorometric detection as previously reported (Sloley et al., 1992). In one experiment, the effects of sex steroids on brain and pituitary GABA synthesis rates were determined. Since the accumulation of GABA following GVG injection is linear, synthesis rate can be estimated by slope analysis (Brodie et al., 1966; Mansky et al., 1982). A previously reported method for estimation of GABA synthesis rate (Mansky et al., 1982) utilized the post-mortem accumulation of GABA as an index of neuronal function in rats. In goldfish, however, post-mortem accumulation of GABA is slow and non-linear (Sloley et al., 1992) and thus, cannot be used for such calculations. Sexually regressed female fish were implanted with blank, P_4, T and E_2, and 5 days later injected with GVG (300 µg/g). Blood, brain and pituitaries were collected immediately after (0 h) and again at 8 and 24 h following GVG. Pituitary protein concentrations were determined by the method of Bradford (1976).

The effect of steroid treatment on taurine-induced GTH release

At various time of the year, the effects of TAU on GTH release *in vivo* were tested in gonad-intact control and steroid-implanted goldfish. On the day of experimentation, TAU (1 mg/g; 10 µl/g BW) was dissolved in 0.6 % NaCl and injected i.p., and blood samples taken 30 min later. In one experiment, the effects of the TAU precursor hypotaurine (HT) were tested in controls and sexually regressed fish that were either untreated or treated for 3 weeks with T. Blood was collected 45 min after HT (1 mg/g; 10 µl/g BW) injection.

The effect of N-methyl- D,L,-aspartate (NMA) on GTH release

Effects of the GLU receptor agonist NMA (2.5-25 µg/g; 10 µl/g BW) were tested in female goldfish in post-spawning condition (GSI=3.5 %). NMA was dissolved in saline-vehicle and injected i.p., and blood was collected 30 min later. The effects of NMA (25 µg/g) were also tested in sexually regressed fish (GSI=1 %). Taurine has been reported to inhibit NMA-stimulated LH release in rats (Price et al., 1978). To test the effects on GTH release in goldfish, 25 µg/g NMA was injected alone or in combination with TAU (1 mg/g) and blood collected 30 min later. The effects of 10 day implantation of P_4, T, and E_2 on NMA-induced (50 µg/g) GTH release were tested in sexually regressed goldfish (GSI=0.9 %) .

The role of catecholaminergic (CA) neurotransmission in mediating the GTH response to GVG, TAU and NMA.

Since the tyrosine hydroxylase inhibitor α-methyl-para-tyrosine (MPT) has been shown to severely deplete brain and pituitary levels of norepinephrine and dopamine in goldfish (Trudeau et al., 1992; Chapter 4), it was used to study the role of catecholamines in GABA and NMA-mediated release of GTH. Goldfish were injected with MPT (240 µg/g) at 4 days and 1 day prior to experimentation. To confirm MPT action, pituitary DA levels were determined by HPLC (Sloley et al., 1991). The effect of the DA receptor antagonist domperidone (DOM; Janssen Pharmaceutica, Beerse, Belgium) on the GTH response to GVG and TAU was also examined in sexually mature female goldfish. In one experiment, DOM (1 µg/g) was injected alone or in combination with GVG (300 µg/g) and blood was collected 24 h later. In a second experiment, DOM (1 µg/g) was injected 24 h before injection of TAU
(1 mg/g); blood was collected 30 min later.

Radioimmunoassay (RIA)

Serum GTH levels were measured using a double antibody RIA (Peter et al., 1984) specific for GTH-II (Van Der Kraak et al., 1992). All samples were assayed in duplicate (10 µl serum). Within and between assay coefficients of variation are <10 % (Trudeau et al., 1991a).

Statistical Analysis

Serum GTH levels were analysed by the least squares method of analysis of variance (AOV; Statistical Analysis Systems, Cary, NC); for experiments examining treatment interactions, 2-way AOV was used. Treatment group means were considered statistically different if $p<0.05$. GABA synthesis rates were estimated from the slope of GABA increase after GVG injection (Brodie et al. 1966; Mansky et al., 1982). To determine significant differences between synthesis rates, Z values were calculated as reported (Rance et al., 1981) and entered in a table of standard normal distribution.

Results

The effects of GABA and GVG on GTH release

The time course of GABA increase following GVG injection is shown in Fig. 5.1. Injection of GVG 300 µg/g elevated brain and pituitary GABA levels within 4 h and continued to increase linearly for at least the next 44 h (i.e. 48 h after injection). Associated with increased GABA levels in brain and pituitary were elevations in serum GTH (Fig.5.2) by 8 h. TAU and GLU levels in brain and PIT were not affected by GVG (data not shown). The effects of sex steroids on the accumulation of GABA in brain and pituitary following GABA-transaminase inhibition with GVG is summarized in Table 5.1. Implantation of P_4 or T significantly decreased the synthesis rate of GABA in the TEL-POA and PIT, but did not in the HYP. In contrast E_2 increased GABA synthesis rate in the HYP and PIT, but did not in the TEL-POA. Sex steroid implantation also affected the GTH response to GVG (Fig. 5.3). Serum GTH increased progressively following GVG injection in blank, P_4 and T treated animals; the magnitude of these responses were not different between treatments. In contrast, GVG was ineffective in stimulating GTH release in E_2 treated goldfish.

The effects of sex steroid implantation on the GTH release-response to GABA (100 µg/g) are shown in Fig.5.4. In sexually regressed goldfish in July (Fig. 5.4A) GABA was ineffective in stimulating GTH release in control and E_2 treated fish. In contrast, GABA stimulated GTH release in T-treated animals. GABA stimulated GTH release in sexually recrudescent goldfish in September (Fig. 5.4B) and T enhanced this response. In contrast, P_4 and E_2 did not affect GABA-responsiveness.

The effects of gonadal steroids on TAU-stimulated GTH release

When injected i.p., TAU (1 mg/g) stimulated GTH release within 30 min in sexually regressed and sexually recrudescent goldfish (Fig. 5.5). In sexually regressed fish, implantation for 5 days with E_2 or T enhanced ($p<0.05$) the GTH response to TAU (Fig. 5.5A). Testosterone implantation for 10 days potentiated taurine-induced GTH release in sexually recrudescent male goldfish (Fig. 5.5B). The TAU precursor, HT did not stimulate GTH release in blank-implanted sexually regressed goldfish, but enhanced GTH secretion in animals treated with T for 3 weeks (Fig. 5.6).

The effects of NMA on GTH release

Injection of 25 μg/g but not 2. μg/g NMA stimulated a significant ($p<0.05$) increase in serum GTH levels at 30 min following injection; serum GTH levels were 10.5 ± 1.8, ng/ml 8.6 ± 0.7 and 19.4 ± 3.3 ng/ml in saline, 2.5 μg/g and 25 μg/g NMA-injected females, respectively. In sexually regressed fish, injection of 50 μg/g NMA stimulated an increase in serum GTH levels and 10 day implantation with P_4, T or E_2 did not affect NMA-stimulated GTH levels (Fig. 5.7). Injection of TAU (1 mg/g) also stimulated an increase in serum GTH release in sexually regressed fish (Fig. 5.8); there was no indication of an interaction of TAU and NMA (Fig. 5.8).

The effects of CA neurotransmission on the GTH response to GVG, TAU and NMA

Pituitary DA levels were 984 ± 185 pg/pit (n=7) and 96 ± 18 pg/pit (n=7) in control and MPT-injected animals (t-test; $p<0.05$), respectively. The effects of MPT on basal and GVG-induced serum GTH levels are shown in Fig. 5.9. Serum GTH levels were increased a small but significant amount at 24 and 48 h after the second MPT injection. Injection of GVG alone increased serum GTH levels approximately 4-5 fold at 24-48 h. Pre-treatment with MPT potentiated the serum GTH response to GVG at 24-48 h. In another experiment, serum GTH levels were elevated by MPT injection whereas injection of NMA alone had no effect on GTH in sexually regressed animals (Fig. 5.10). Serum GTH levels in fish injected with both MPT and NMA were significantly higher ($p<0.05$) than controls but only slightly ($p>0.05$) higher than that in fish injected with MPT alone (Fig. 5.10). Effects of the DA receptor antagonist, DOM on the GTH response to TAU and GVG are shown in Fig. 5.11. DOM alone did not affect serum GTH levels whereas TAU (Fig. 5.11A) and GVG (Fig.5.11B) stimulated GTH release. Co-administration of DOM with either TAU or GVG did not further increase serum GTH levels.

Discussion

GABA, GLU and TAU are present in the teleost preoptico-hypophyseal axis (Sloley et al., 1992) and results of the present study implicate them in the neuroendocrine control of GTH release in the goldfish. Injection of GABA, TAU or the GLU receptor agonist NMA, results in GTH release within 30 min suggesting that these substances can act rapidly to affect hypophyseal hormone secretion in the goldfish, similar to mammals (Price et al.,1978; Schiebel et al., 1981; McCann and Rettori, 1988; Bourguignon et al., 1989).

GABA has been shown to stimulate GnRH release from goldfish pituitary slices in vitro; however, it has no direct action on gonadotroph cells (Kah et al., 1992). Since DA is a potent inhibitor of GTH (Peter et al., 1986) and GnRH (Yu and Peter, 1990; Yu et al., 1991) release in goldfish, we hypothesized that GABA may also act to inhibit DA neuronal function, similar to the situation in mammals, (Anden and Stock, 1973; Mansky et al., 1982), and thereby leading to a stimulation of GTH release. Severe depletion of brain and pituitary DA and NE by inhibition of catecholamine synthesis with MPT is capable of stimulating GTH release in goldfish (Chang et al., 1983; Trudeau et al., 1992; Chapter 4) and this was confirmed in the present studies. We reasoned that if GABA was acting to inhibit dopaminergic function then the release of GTH induced by the GABA transaminase inhibitor GVG, should be blocked or reduced in goldfish injected with MPT. On the contrary, pretreatment with MPT potentiated GVG-induced GTH release. In addition, domperidone, a DA receptor antagonist with specific actions at the pituitary (Omeljaniuk et al.,1988; Sloley et al., 1992) did not block GVG action. Furthermore, GVG does not affect brain and pituitary DA levels in goldfish (Sloley et al., 1992). These results suggest that GABA does not inhibit DA neuronal function to stimulate GTH release.

One novel aspect of the present studies is the demonstration of a stimulatory effect of TAU on GTH secretion, confirming our preliminary findings (Sloley et al., 1992). This is contrary to the usual inhibitory role of TAU in mammalian systems (McGeer and McGeer, 1989; Huxtable, 1989). In mammalian studies TAU and GABA are frequently co-localized (i.e., cerebellar Purkinje cells; Ottersen et al., 1988) and often share similar inhibitory actions on DA neurotransmission (Anden and Stock, 1973; Biswas and Carlson, 1977; Ahtee and Vahala, 1985; Kontro, 1987). However, as with GVG stimulated GTH release, TAU action on GTH release in goldfish is potentiated by MPT pretreatment (Sloley et al., 1991a) and TAU action was not affected by DOM (present study). Thus, the present results demonstrate that the stimulatory actions of TAU on GTH release in goldfish do not appear to involve inhibition of the dopaminergic system. A major question not addressed by the present study is the cellular localization of TAU within the brain and pituitary, which would give insight into the site and mechanisms of TAU action on GTH release. In the rat, TAU is ubiquitously present in neural tissues and glial elements and is considered to have predominantly a neuromodulatory rather than a neurotransmitter function (Huxtable, 1989; Schousboe et al., 1990). In this regard, TAU has been assigned a neuromodulatory role in the control of prolactin secretion in the rat (Schiebel et al., 1981). Taurine action on GTH release may be a result of its effects on cellular calcium and potassium ion distribution, and/or membrane phospholipid availability (Huxtable, 1989), since these factors would be expected to affect GnRH and/or GTH release (Hawes and Conn, 1990; Kordon and Drouva, 1990; Yu et al., 1991).

The role of the amino acid neurotransmitter GABA, in mediating steroid negative feedback has received considerable attention (Tappaz et al., 1982; Wuttke et al., 1987; McCann and Rettori, 1988). A large proportion of E_2-concentrating neurons in the HYP of the rat are GABAergic and GABA acts within the preoptic area to modulate CA neurotransmission thereby controlling LH secretion (Mansky et al., 1982; Wuttke et al., 1987; Herbison et al., 1990). We have demonstrated that GABA may be important in the stimulation of GTH secretion in the goldfish and that its actions are steroid-dependent. Since GVG is very effective in blocking GABA transaminase activity, the time-dependent accumulation of brain and pituitary GABA levels is likely a reflection of ongoing GABA synthesis within GABAergic neurons (Brustle et al., 1988; Loscher et al., 1989). In the goldfish it appears that E_2 can increase GABA synthesis in the HYP and PIT (present results) and can either inhibit (Kah et al., 1992) or have no effect on GABA-induced GTH secretion. Furthermore, E_2 blocks the stimulatory effect of GVG on GTH release in sexually regressed goldfish. This is, in effect, a negative feedback action of E_2. On the other hand, T decreased the rate of GABA synthesis in the TEL-POA and PIT without affecting GABA in the HYP and T potentiated GABA-stimulated GTH release. This is, in part, a positive feedback action of T. Progesterone also decreased GABA synthesis rate in the TEL-POA and PIT, but did not affect HYP GABA or pituitary responsiveness to injected GABA. However, the results are somewhat difficult to interpret since changes in PIT GABA synthesis and GTH responsiveness to GABA or GVG injection were inversely related. However, since sex steroids can modulate the activity of the GABA-synthesizing enzyme, glutamic acid decarboxylase (McGinnis et al., 1980; Wallis and Luttge, 1980) as well as GABA receptor numbers (Lasaga et al., 1988; Schumacher et al., 1989) in neural tissues, differential responses of neurotransmitter-synthesising and receptor mechanisms could be the basis for our observations. Indeed, it has been shown that E_2 can increase hypothalamic glutamic acid decarboxylase activity (Duvilanski et al., 1983) as well as reducing hypothalamic GABA receptor numbers (Schumacher et al., 1989). That GABA is localized (Kah et al.,1987; Martinoli et al.,1990) in brain areas that also concentrate T and E_2 (Kim et al., 1978) in the goldfish further implicate this amino acid in mediation of sex steroid feedback.

Recent evidence derived primarily from studies in the rat , indicate that GLU acting via hypothalamic NMDA-, kainate- or quinolinate-type excitatory receptors, stimulates GnRH and LH secretion (Nemeroff et al., 1985; Bourguignon et al., 1989; Brann and Mahesh, 1991). In the primate, NMA is capable of activating a quiescent hypothalamo-hypophyseal complex during the prepubertal phase (Plant, 1988). We reasoned that if NMA has similar actions in the goldfish, then it should stimulate GTH release in sexually regressed fish, when serum and pituitary GTH, pituitary responsiveness to GnRH, gonadal size and sex hormone levels are all reduced (Kobayashi et al., 1986; Trudeau et al., 1991). A stimulatory action of 25 μg/g NMA was found in goldfish in post-spawning condition, and by a dosage of 50 μg/g but not 25 μg/g in fish in the sexually regressed phase. Implantation of sex steroids in regressed fish, which results in a highly potentiated GnRH response (Trudeau et al., 1991), did not affect NMA action on GTH release. That glutamate (Sloley et al., 1992) and NMA are less effective in stimulating GTH release in goldfish that are highly responsive to TAU, GABA or GnRH suggests that NMA-mediated control of GTH may represent a minor component of the neuroendocrine mechanisms governing GTH release in this species. Confirmation of this hypothesis, however, awaits more extensive experimental evidence.

In summary, GABA, TAU and NMA stimulate GTH release *in vivo* in the goldfish. Treatments with GVG increased brain and pituitary GABA levels and also stimulated GTH release. GABA and TAU stimulated GTH release is enhanced by pretreatment with MPT and sex steroids. In contrast, NMA has relatively little effectiveness in stimulating GTH release and its effects are not enhanced by sex steroids. GABA neurons of the goldfish are sensitive to sex steroids and this could be part of the mechanism of feedback regulation of GTH secretion in teleosts.

Table 5.1. The effects of 5 day implantation of P_4, T and E_2, on GABA concentrations and GABA synthesis rates in the TEL-POA, HYP and PIT of sexually regressed goldfish. Data are presented as mean ± SEM. Calculation procedures are described in Materials and Methods.

	Blank	**P_4**	**T**	**E2**
TEL-POA				
concentration (µg/g)	202.8±12.8	202.8±16.9	203.4±23.0	205.6±19.5
synthesis rate (µg/g/h)	29.5± 2.1	19.8± 1.9**	21.9± 1.7**	27.5±2.1
HYP				
concentration (µg/g)	168.1± 8.1	170.4± 7.6	169.3± 5.0	180.6± 9.3
synthesis rate (µg/g/h)	25.0± 1.6	26.5± 1.9	25.4± 1.8	33.5±1.7**
PIT				
concentration (ng/mg protein)	383.2±44.3	358.0±44.1	343.2±22.5	439.4±39.2
synthesis rate (ng/mg protein/h)	90.0±4.1	67.6± 6.1**	76.8± 5.1**	114.4±13.8*

(*)-$p<0.05$ versus control; (**)-$p<0.01$ versus control.

Synthesis rate is the slope of GABA accumulatioin following GVG (300 µg/g) injection (n=11-12 for 0, 8 and 24 h).

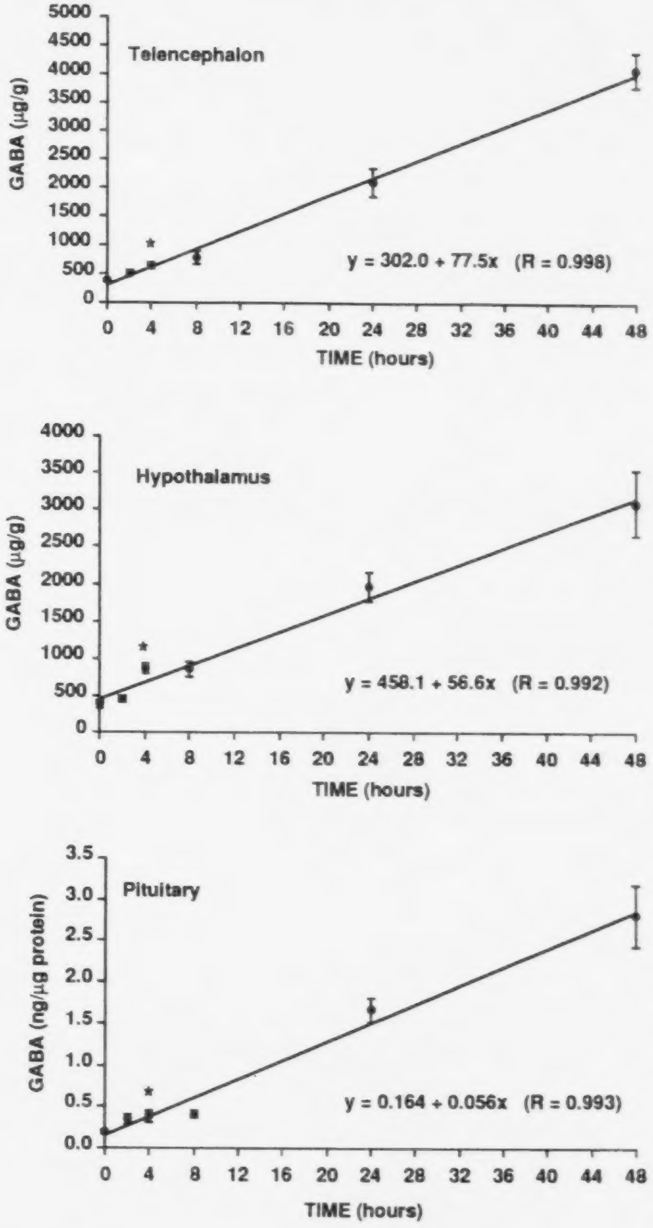


Fig. 5.1. Time course of the effects of GVG (300 μg/g) on GABA concentrations in the TEL-POA (upper panel) HYP (middle panel) and PIT (lower panel) of postspawning goldfish in June (GSI=1.9%). Data are mean ± SEM (n=12). (*) indicates the first significant increase in GABA concentrations ($p<0.05$) compared to preinjection levels. Linearity of GABA accumulation is indicated by highly significant correlation coefficients (R).

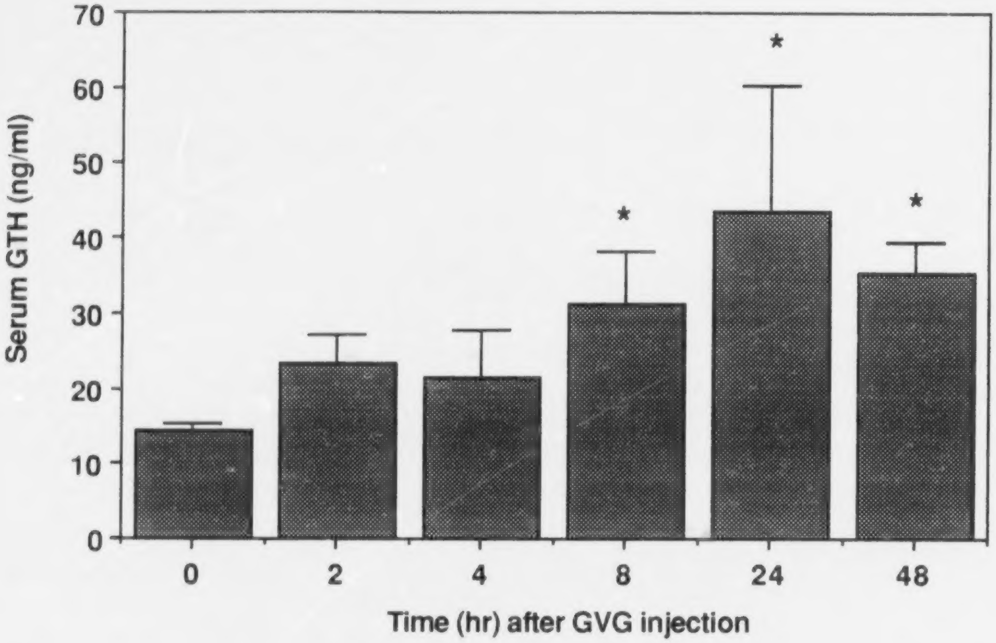


Fig. 5.2. Time course of the effects of GVG (300 μg/g) on serum GTH levels in postspawning goldfish in June (GSI=1.9%). Data are mean ± SEM (n=12). (*) indicates a significant increase in GTH levels ($p<0.05$) compared to preinjectiion levels.

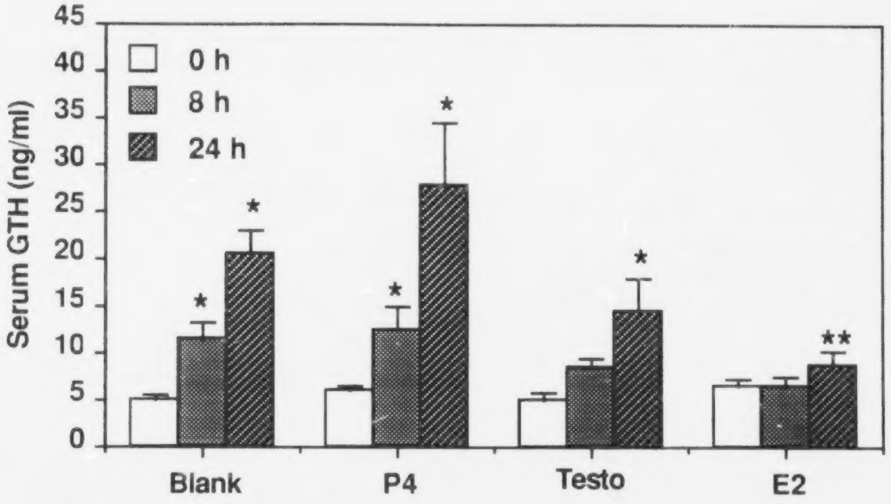


Fig. 5.3. The effects of 5 day implantion of progesterone (P_4), testosterone (T) and estradiol (E_2) on the serum GTH response at 0, 8 and 24 h following GVG injection (300 µg/g) in sexually regressed goldfish in August (mixed sex; GSI=1 %). Data are mean ± SEM (n=11-12). (*) indicates a significant effect ($p<0.05$) of GVG versus 0h. (**) indicates that GVG did not stimulate GTH release in E_2-treated fish ($p>0.05$).

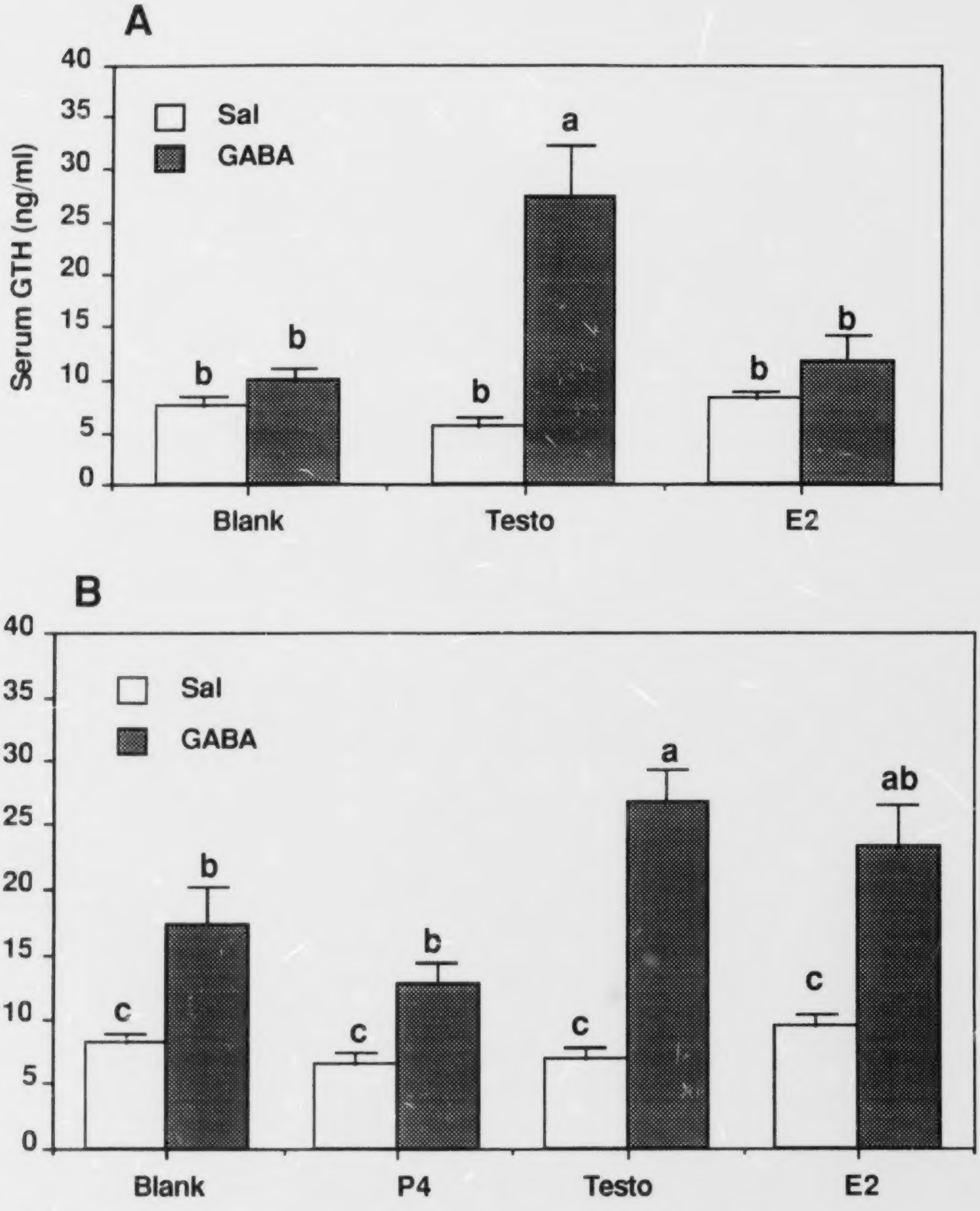


Fig. 5.4. **(A)**. The effects of 10 day implantation of T and E_2 on the serum GTH responses to GABA (100 µg/g; 30 min) in (A) sexually regressed goldfish July; GSI= 1%; mixed sex). **(B)** The effects of 5 day implantation of P_4 ,T and E_2 on the serum GTH responses to GABA (100 µg/g) in sexually recrudescent goldfish (Sept; GSI=1.2 %; mixed sex). Data are mean ± SEM (n=10-12).

^{a,b,c}Means with different superscripts are significantly different ($p<0.05$).

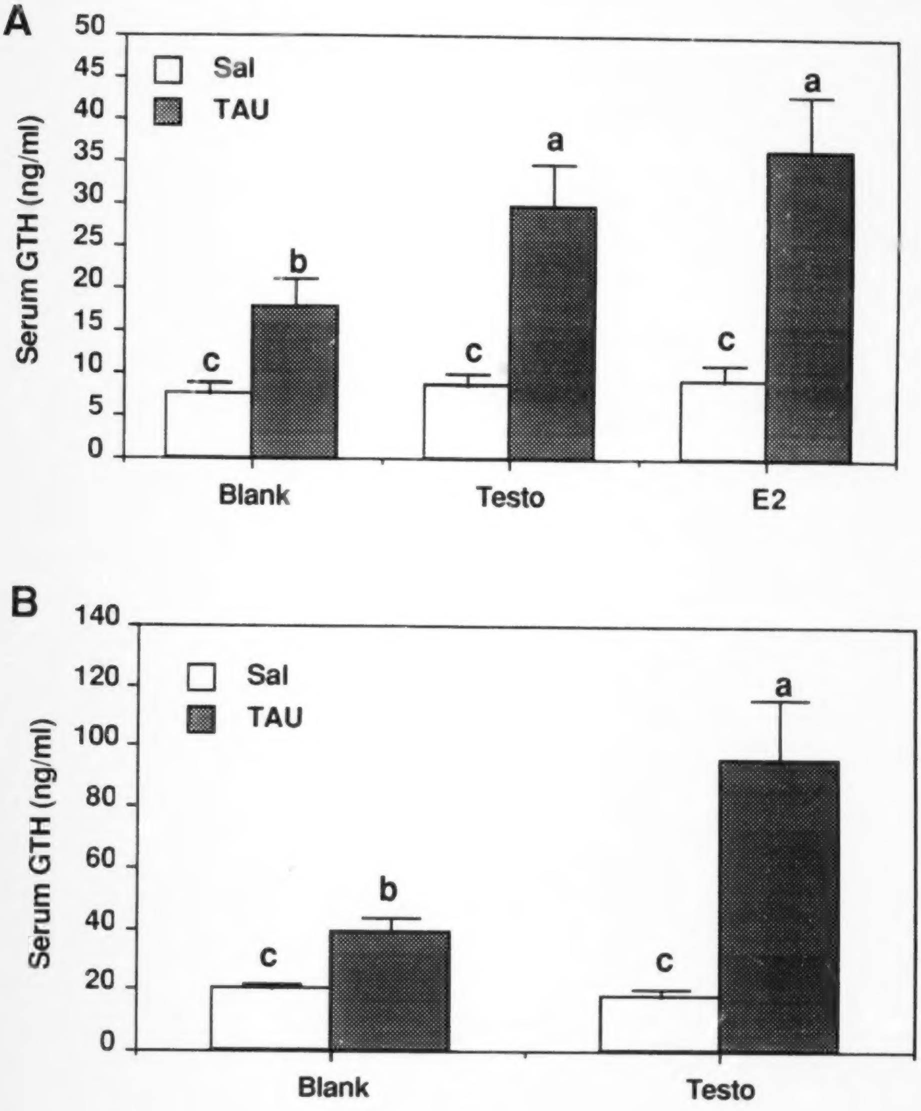


Fig. 5.5. (**A**) The effects of 5 day implantation of T and E_2 on the serum GTH response to TAU (1 mg/g; 30 min.) in sexually regressed female goldfish (August; GSI= 1%). (**B**) The effects of 10 day implantation of T on the serum GTH response to TAU (1 mg/g; 30 min.) in sexually recrudescent male goldfish (February; GSI= 1%). Data are mean ± SEM (n=10-12).

^{a,b,c}Means with different superscripts are significantly different ($p<0.05$).

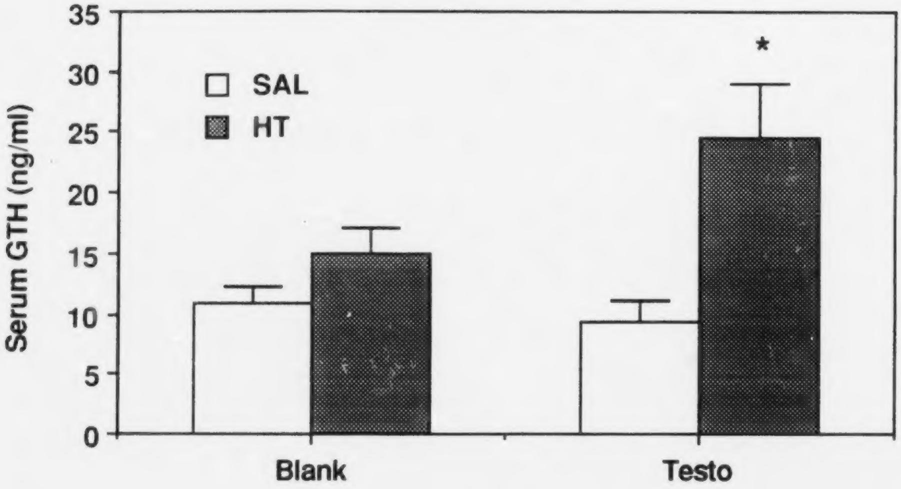


Fig. 5.6. The effects of 3 week implantation of T on the serum GTH response to HT (1 mg/g; 45 min.) in sexually regressed goldfish (July; GSI= 1.2%). Data are mean ± SEM (n=12). (*) indicates a significant ($p<0.05$) effect of HT on GTH release in T-treated animals.

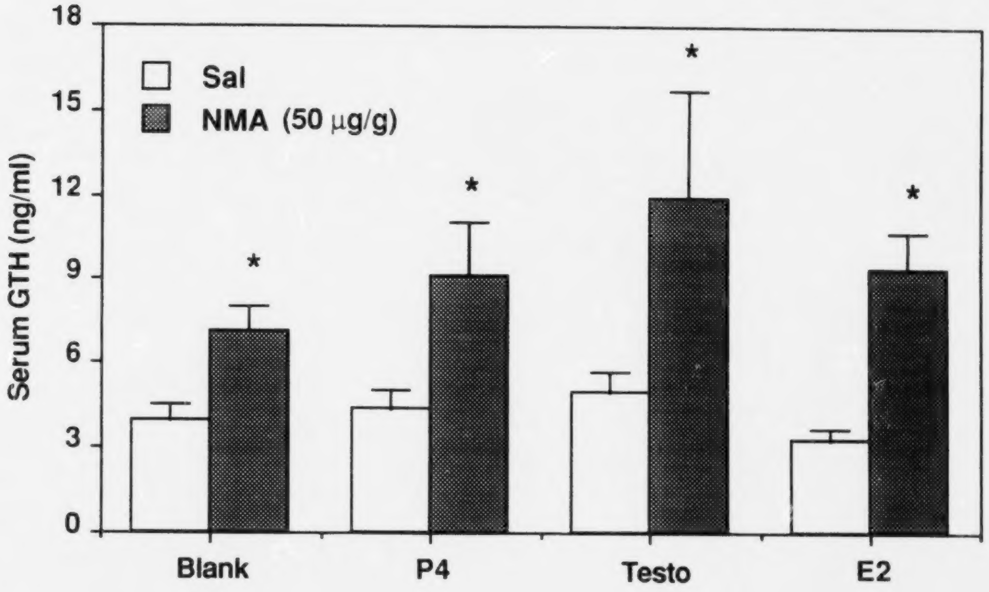


Fig. 5.7. The effects of 10 day implantation of P_4 ,T and E_2 on the serum GTH response to NMA (50 µg/g) in sexually regressed goldfish (August; GSI= 1%; mixed sex). Data are mean ± SEM (n=10-12). (*) indicates a significant ($p<0.05$) effect of NMA. Sex steroids did not affect NMA-induced GTH release.

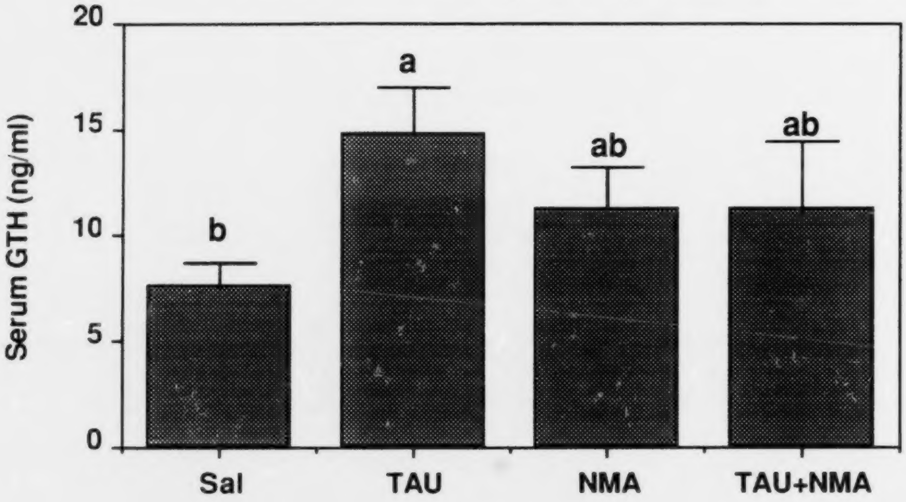


Fig. 5.8. The effects of NMA (25 µg/g) on TAU (1 mg/g)-induced GTH release (30 min.) in sexually regressed goldfish (July; GSI=1.2%) Data are mean±SEM (n=15). ^{a,b}Means with different superscripts are significantly different ($p<0.05$).

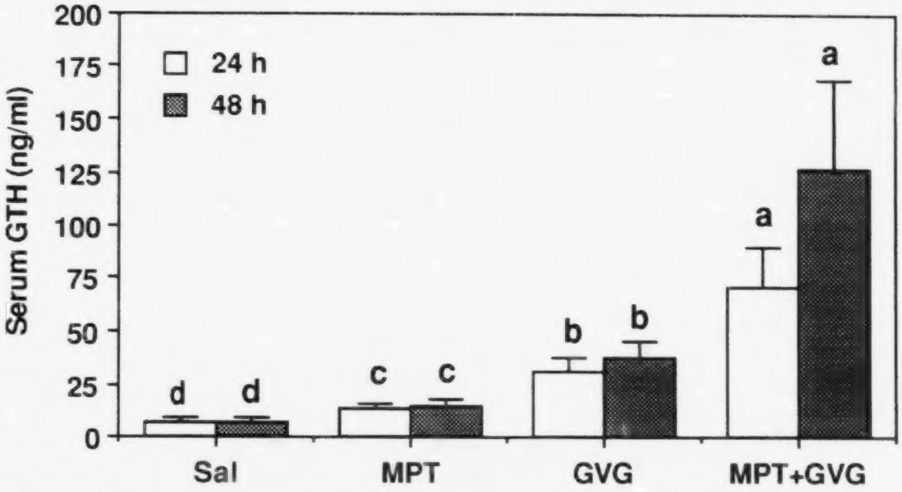


Fig. 5.9. The effect of MPT (240 μg/g 4 and 1 day prior to experimentation) injection on serum GTH 24 and 48 h following GVG (300 μg/g) in sexually regressing female goldfish (August; GSI=2%). Data are mean ±SEM (n=10-12).

^{a,b,c,d}Means with different superscripts are significantly different ($p<0.05$).

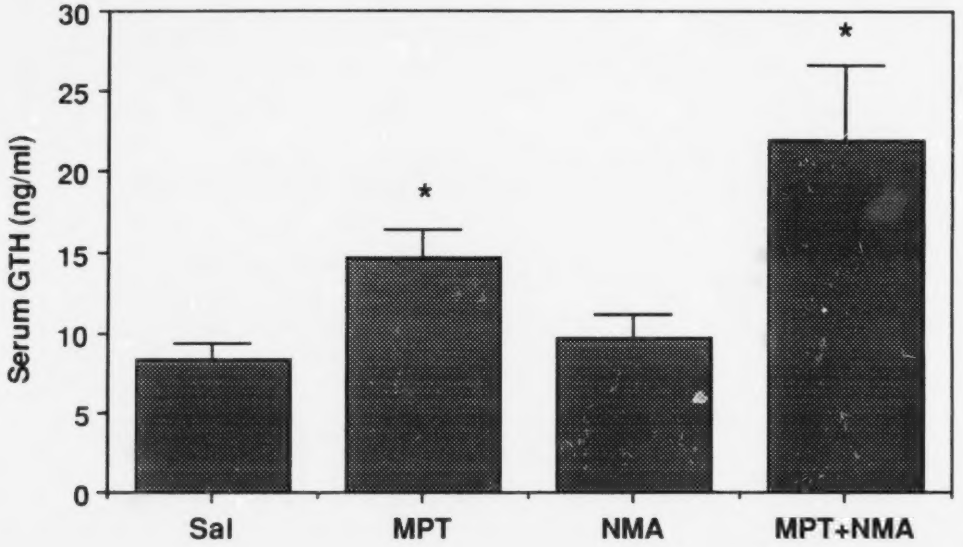
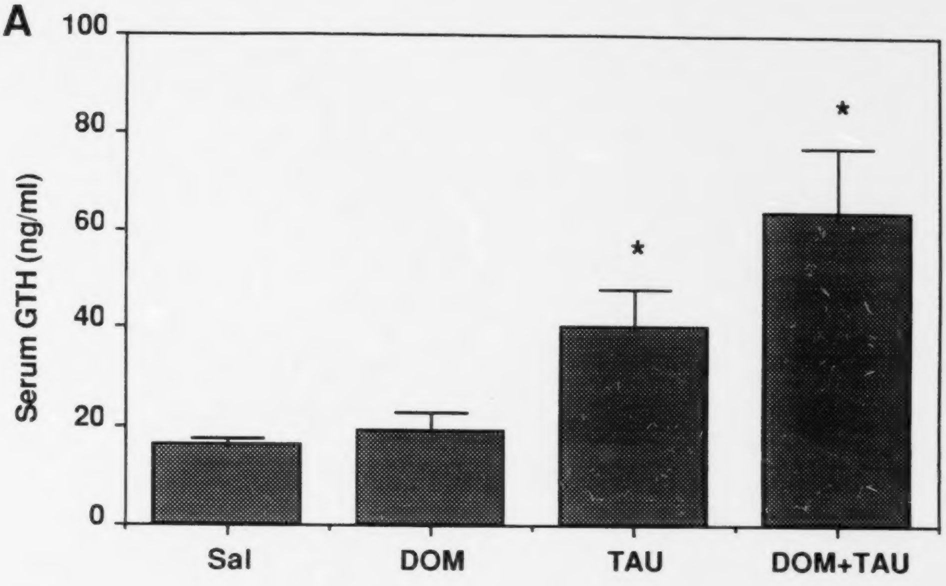


Fig.5.10. The effect of MPT (240 µg/g 4 and 1 day prior to experimentation) injection on serum GTH response to NMA (25 µg/g).in sexually regressed goldfish (Sept; GSI=1.3%). Data are mean ±SEM (n=10-12). (*) indicates a significant ($p<0.05$) effect of MPT. NMA was without effect and MPT pretreatment did not alter NMA action.



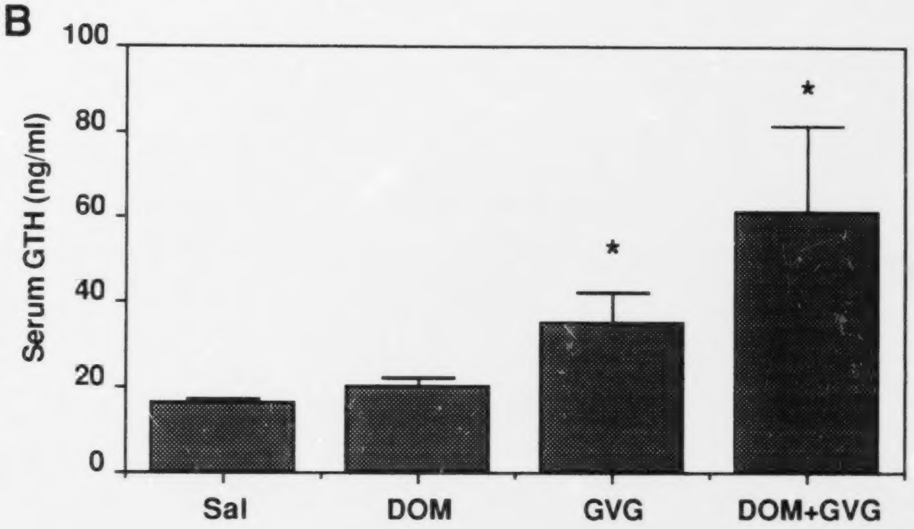


Fig. 5.11. The effect of DOM (1 µg/g) on the serum GTH response to (A) TAU (1 mg/g) and (B) GVG (300 µg/g) in sexually recrudescent female goldfish (April; GSI=2.2%). Data are mean± SEM (n=12). The (*) indicates a significant elevation of GTH following injection of TAU or GVG. DOM pretreatment did not affect this response.

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6. INTERACTIONS OF ESTRADIOL WITH GONADOTROPIN-RELEASING HORMONE AND THYROTROPIN-RELEASING HORMONE IN THE CONTROL OF GROWTH HORMONE SECRETION IN THE GOLDFISH*

Introduction

A role for gonadal steroids in the control of growth hormone (GH) secretion has been suggested since there is a marked sexual dimorphism in GH secretory patterns in mammals (Jansson et al., 1985; Thorner et al., 1986). This sexual dimorphism in GH secretion is dependent on sex steroids; gonadectomy with replacement of testosterone (T) or estradiol (E_2) restores typical male or female GH secretory patterns, respectively (Jansson et al., 1985). Furthermore, sex steroids can alter GH responsiveness to stimulatory neuropeptides. In this regard, it is generally accepted that T enhances and E_2 reduces basal and growth hormone-releasing factor (GRF)-induced GH secretion in rats (Jansson et al., 1985; Hertz et al., 1989).

In other vertebrate species very little is known about the role of sex steroids in control of GH secretion. Chicken pituitary glands incubated *in vitro* with T, E_2 or progesterone have a reduced GH response to thyrotropin-releasing hormone (TRH), and no apparent effect of steroid treatment on basal GH secretion (Hall et al., 1984 a,b,c). *In vivo,* T reduces plasma GH in chickens and turkeys (Harvey, 1983). In teleost fish, it has been suggested that GH secretion may be influenced by sex steroids since pharmacological treatments with E_2 or T affect sommatotroph morphology in eels (Olivereau and Olivereau, 1979). Furthermore, androgens and estrogens stimulate somatic growth in a number of fish species (see Donaldson et al., 1979 for review).

Both GRF (Parker and Sherwood, 1990;Vaughan et al., 1991) and somatostatin (SRIF) (Hobart et al., 1980) have been isolated from teleost sources and have actions controlling GH secretion in fish (Marchant et al., 1987; Luo and McKeown, 1989) as in mammals (Muller, 1987). In addition, salmon gonadotropin-releasing hormone (sGnRH) and chicken gonadotropin-releasing hormone-II (cGnRH-II), the endogenous GnRHs in

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(*)- A version of this chapter has been accepted for publication. Trudeau, Somoza, Nahorniak and Peter. Neuroendocrinology : in press.

goldfish (Yu et al., 1988), stimulate GH as well as GTH secretion (Marchant et al., 1989a; Chang et al., 1990) GnRH-induced GH but not GTH secretion is inhibited by SRIF (Marchant et al., 1989a). The stimulatory action of NPY on GH release has been characterized in goldfish (Peng et al.,1990). Dopamine (DA), and norepinephrine (NE) respectively stimulate and inhibit GH secretion in the goldfish, both *in vivo* (Chang et al., 1985) and *in vitro* (Chang et al., 1990; Peter et al., 1990).

Another factor known to stimulate GH secretion in many vertebrate species is the tripeptide TRH (see Harvey, 1990 for review). Best characterized are the effects of TRH on GH secretion in birds where it is considered a major stimulatory factor (Harvey 1983; 1990). A stimulatory effect of TRH on GH secretion has been suggested for teleosts, but these data are not clear and are based on electrophoretic measurements of GH secretion (e.g. sailfin molly: Wigham and Batten 1984).

In the present paper, we examined the effects of T and E_2 treatment on serum GH levels in the female goldfish throughout the reproductive cycle. Data are presented that suggest that E_2 but not T stimulates GH secretion *in vivo* in the goldfish. One possible mechanism whereby E_2 increases GH release is via enhanced response to stimulatory neuropeptides. The effect of *in vivo* E_2 treatment on the *in vitro* GH release response to a GH-releasing factor, salmon GnRH (sGnRH), was also evaluated. Furthermore, the effects of TRH on *in vitro* GH release were characterized. The data demonstrate that sGnRH- and TRH-stimulated GH secretion is enhanced by E_2 pretreatment.

Materials and methods

Animals

Common or comet varieties of goldfish (*Carassius auratus*) were purchased throughout the year from commercial suppliers (Grassyforks Fisheries; Martinsville, Indiana, and Ozark Fisheries, Stoutland, Missouri, U.S.A.). Seasonal variations in serum gonadotropin (GTH), gonadosomatic index (gonad weight as a percentage of total body weight; GSI) and serum sex steroid levels have been described previously (Trudeau et al, 1991). Animal maintenance and feeding protocols have also been reported (Trudeau et al., 1991).

Steroid treatment and blood collection

Solid silastic pellets containing T, E_2 or no steroid (Blank) were manufactured and implanted intraperitoneally as previously described (Trudeau et al., 1991). Fish received a steroid dose of 25-100 μg/g body weight.

At various times of the year, female fish were implanted with silastic pellets, and 5 or 10 days later blood was collected from anaesthetized (tricaine methane sulfonate) animals by caudal puncture using 25 G needles to assess the effects of steroid treatment on serum GH. Blood was allowed to clot overnight (at 4°C), centrifuged and serum was kept frozen (-20°C) until hormone analysis.

Experimental Protocols

Effects of sGnRH on GH release in vitro

The effects of *in vivo* E_2 treatment on *in vitro* hormone release from pars distalis (p.d.) fragments of the goldfish pituitary were examined using an established perifusion system (Marchant et al., 1987; Peng et al., 1990).

In experiment 1, the effect of E_2 on sGnRH-induced GH secretion *in vitro* was examined in sexually regressed female fish in July (GSI=2 %). Females were implanted for 5 days with E_2 (100 μg/g body weight) or blank pellets. Following dissection, p.d. fragments were preincubated in perifusion for 2 hours prior to experimentation. Fragments were exposed at hourly intervals to 2-minute pulses of 0.5, 5 and 50 nM sGnRH. Medium effluent was collected at 5 minute intervals. The mean GH levels of the 6 samples (30 minutes) preceeding a sGnRH pulse were considered representative of basal secretion.

A second experiment examining the effects of E_2 was carried out in October using female fish in early stages of sexual recrudescence (GSI=3 %). The *in vitro* protocol was as described above except that the doses of sGnRH tested were 1, 10, and 100 nM.

Effects of TRH on GH release in vitro

Sexually mature female goldfish (April, GSI=9 %) were used to establish the effects of TRH on GH and GTH secretion. Pars distalis fragments were incubated overnight and on the day of experimentation, fragments were exposed to 10 minute pulses of TRH at hourly intervals. Both increasing (0.1-10000 nM) or decreasing (10000-0.1 nM) TRH dose responses were examined. Medium effluent was collected at 10 minute intervals. The mean GH levels of the 3 samples (30 minutes) preceeding a TRH pulse were considered representative of basal secretion. Since 10 minute exposure to TRH may cause down regulation of the GH response, 2 minute pulses of increasing concentrations of TRH (0.1-1000 nM) at hourly intervals were used in a second series of experiments. Medium effluent was collected at 10 minute intervals. TRH dose response curves were established for sexually regressed (August, GSI= 1 %) and sexually mature (March-April, GSI=9 %) female goldfish.

The effect of estradiol on TRH-induced GH secretion *in vitro* was examined in sexually recrudescent (October, GSI= 2 %) female fish. Females were implanted for 5 days with E_2 or blank as described above. Fragments were exposed to 2-minute pulses of increasing doses of TRH (0.1, 30 and 100 nM) or sGnRH (5 nM). Medium effluent was collected at 5 minute intervals.

Radioimmunoassays (RIA)

GH concentrations in serum and perifusion medium were determined by double antibody RIA as previously described (Cook et al., 1983; Marchant et al., 1989b). GTH concentrations in perifusion medium were determined using a double antibody RIA (Peter et al., 1984) specific for GTH-II (VanDerKraak et al., 1992).

Data analysis

GH secretion *in vitro* was quantified as total amount of hormone (ng) released above basal hormone release or as percentage above basal release. An increment of GH following sGnRH or TRH application was included as part of a response if it was greater than 1 S.E.M. above basal secretion. Following neuropeptide application, GH levels typically returned to basal within 10-15 min. Data were analysed by the least squares method of analysis of variance (Statistical Analysis Systems, Inc., Cary, N.C). Dose response curves for TRH were analysed using the Allfit computer program (DeLean et al., 1978). Estimates of ED_{50} in these experiments were compared statistically using 95% confidence intervals.

Results

The effects of T and E_2 implantation on serum GH concentration

Serum GH levels in blank implanted control animals varied seasonally (Tables 6.1 and 6.2). Serum GH levels increased from April to May and declined progressively through June (see Table 6.2, control values) and July. Serum GH concentrations increased again in October. In the following year, GH was highest during the period from February to May and declined to intermediate levels in October 1989. As shown in Table 6.1, the effects of E_2 were tested throughout the experimental period and were found to consistently increase serum GH ($p<0.05$); GH levels in E_2 treated fish were approximately 2-4 times those in control fish. In contrast, T implantation did not affect GH at any time of the year (Table 6.1). In another study in October, we examined whether longer implantation (10 days) of E_2 and T would affect serum GH. Similar to the results obtained with 5 day implantations, E_2 but not T stimulated GH secretion following 10 days implantation; GH levels were 77.9 ± 11.2 (n=9), 326.7 ± 23.5 (n=10) and 63.8 ± 12.8 (n=8) ng/ml for blank, E_2 and T implanted female fish, respectively. We also performed one experiment with sexually regressed male fish in July and found that 5 day implantation of 100 µg/g E_2 elevated GH; serum concentrations of GH were 22.8 ± 2.9 (n=4) and 131.6 ± 66.8 (n=3) ng/ml for blank and E_2 treated males, respectively.

The effects of various doses of E_2 on GH secretion were tested in post-spawning female fish in June. Although there was no clear dose response relationship, graded doses of E_2 progressively increased serum GH (Table 6.2).

The effect of in vivo E_2 treatment on in vitro GH response to sGnRH

The effects of *in vivo* E_2 treatment on *in vitro* GH responsiveness to sGnRH are shown in Figs. 6.1 and 6.2. In experiment 1 (Fig. 6.1), p.d. fragments from E_2 treated fish had a higher basal GH secretion over the first 30 minutes of perifusion than controls (192 ± 10 ng/ml vs 43 ± 5 ng/ml; $p<0.05$). sGnRH stimulated GH release in a dose-dependent manner from p.d. fragments of blank implanted control animals (Fig. 6.1). In contrast, the lowest dose (0.5 nM) of sGnRH did not elicit GH release above basal in p.d. fragments obtained from E_2 treated fish (Fig. 6.1). Total GH release in response to 5 and 50 nM sGnRH, however, was significantly ($p<0.05$) higher from E_2 treated fish compared to controls. When the release response data were expressed as % above basal secretion (Fig. 6.1; lower panel), the stimulatory effect of E_2 on sGnRH-induced GH secretion was not apparent.

In experiment 2 (Fig. 6.2), p.d. fragments from E_2 treated fish had a higher basal GH secretion over the first 30 minutes of perifusion than blank implanted controls (105 ± 5 ng/ml vs 39 ± 11 ng/ml; $p<0.05$). sGnRH stimulated GH release in a dose-dependent manner from p.d. fragments of controls (Fig. 6.2). Compared to controls, total GH released above basal was significantly higher ($p<0.05$) from p.d. fragments of E_2 treated fish in response to 1 and 10 nM sGnRH, and only slighly higher ($p>0.05$) at 100 nM sGnRH (Fig. 6.2; upper panel). When the release response data were expressed as % above basal secretion (Fig.6. 2; lower panel), the stimulatory effect of E_2 on sGnRH-induced GH secretion was not apparent.

The effect of TRH on GH secretion from pars distalis fragments in vitro

GH release following 10 minute pulses of increasing or decreasing doses of TRH are shown in Fig. 6.3. Increasing doses of TRH stimulated GH release, but no clear dose-response relationship was observed using 10 minute pulse exposures to TRH (Fig. 6.3A). TRH did not affect GTH secretion. At the end of the perifusion period, 100 nM sGnRH stimulated both GH and GTH release. Decreasing doses of 10 minute pulses of TRH stimulated GH release, but no clear dose-response relationship was observed (Fig. 6.3B). As in the previous experiment, TRH did not affect GTH secretion and 100 nM sGnRH stimulated release of both GH and GTH. Total ng GH released above basal secretion was quantified in these experiments (Fig. 6.3C). The amount of GH released by 10-1000 nM TRH was similar whether TRH was administered in increasing or decreasing order. At 10000 nM, however, GH release was approximately 3-fold lower ($p<0.05$) in columns exposed to increasing doses of TRH. These data indicate that p.d. fragments exposed to 10 minute pulses of high doses of TRH may have a down-regulated GH response. To avoid possible down-regulation in subsequent experiments, lower range dose responses (0.1 1000 nM) and 2-minute TRH pulses were used (see next paragraph). The GH release response to 100 nM sGnRH was similar ($p>0.05$) in columns previously exposed to increasing or decreasing doses of TRH.

Pars distalis fragments obtained from sexually regressed and sexually mature female goldfish responded, in a dose-dependent manner, to 2-minute pulses of increasing doses of TRH (Fig. 6.4). Basal secretion was higher from p.d. fragments of sexually mature than for sexually regressed female fish (31 ± 6 ng/ml vs 15 ± 3 ng/ml; $p<0.05$). The ED_{50} estimate of TRH-induced GH secretion for regressed females was higher ($p<0.05$) than that in mature females (5.7 ± 3.1 nM vs. 0.53 ± 0.28 nM).

The effect of in vivo E_2 treatment on in vitro GH response to TRH

Pars distalis fragments from E_2 treated sexually regressed fish had higher basal secretion over the first 30 minutes of perifusion than p.d. fragments of blank implanted controls (16 ± 1 ng/ml vs. 6 ± 2 ng/ml; $p<0.05$). TRH stimulated GH release in a dose-dependent manner from p.d. fragments of control fish (Fig. 6.5). Total GH released above basal levels in response to the 3 doses of TRH was significantly higher ($p<0.05$) from p.d. fragments of E_2 treated fish than from controls (Fig.6.5; upper panel). Total GH release in response to 5 nM sGnRH was also significantly higher ($p<0$ .05) from p.d. fragments of E_2 treated fish compared to controls. When the release response data were expressed as %

above basal secretion, the stimulatory effects of E_2 on TRH- and sGnRH-induced GH secretion were not apparent (Fig.6. 5; lower panel).

Discussion

Seasonal variations in serum GH concentrations in female goldfish were evident in the present experiment and were similar to previous studies (Marchant and Peter, 1986). GH levels were higher in late winter and early spring, a period when gonadal size and sex steroid secretion are increasing (Kobayashi et al., 1986; Trudeau et al., 1991). Treatment of female fish with E_2 consistently elevated serum GH whereas T was without effect. In some instances, GH levels in E_2 treated animals greatly exceeded maximal seasonal GH levels in blank implanted control fish suggesting that the estrogenic stimulation of GH is pharmacological. However, serum E_2 levels (5-10 ng/ml) following implantation of 100 μg/g E_2 in silastic (Trudeau et al., 1991) are similar to ovulatory levels in female goldfish (Kobayashi et al., 1987). In addition, lower doses of E_2 also raise serum GH (present study), suggesting a physiological response.

Among the vertebrates, teleost fish are unique in that the adenohypophysis is directly innervated by aminergic and peptidergic neurosecretory neurons (Ball, 1981; Peter et al., 1990). GnRH perikarya from the ventral preoptic area innervate the proximal pars distalis, where gonadotroph and somatotroph cells are located (see Kah, 1986 for review). Very little is known about the brain distribution of TRH in teleosts. In carp, TRH perikarya were found only in the nucleus recessus lateralis of the hypothalamus (Hamano et al., 1990). TRH-immunoreactive fibres were found predominantly in the neural lobe, adjacent to the intermediate lobe of the pituitary. These authors (Hamano et al., 1990) indicated that TRH fibres were rarely visualized in the pars distalis of the pituitary but some fibres, localized in the neural lobe, were in close proximity to the anterior lobe. The physiological function of TRH in fish is not well understood (Jackson and Bolaffi, 1983) but has been shown to stimulate α–melanocyte stimulating hormone secretion from goldfish neurointermediate lobe fragments (Omeljaniuk et al., 1989). Given the relative lack of TRH in or in close proximity to the pars distalis, the potential role of TRH in regulation of teleost adenohypophyseal function is not clear. Using electrophoretic measurement of GH, a single concentration of TRH (approx. 260 nM) was shown to stimulate GH secretion from whole pituitaries of the sailfin molly after 18 hours incubation *in vitro* (Wigham and Batten, 1984). TRH has also been shown to stimulate PRL release from the pars distalis of tilapia (Grau and Helms, 1990). We have clearly demonstrated that in perifusion, short pulses (2 min) of TRH can stimulate GH secretion directly from p.d. fragments of the goldfish. TRH is about equipotent with sGnRH (ED_{50}=2.5 nM; Matchant et al., 1989a) and NPY (ED_{50}= 0.5 nM; Peng et al., 1990) in stimulating GH release in goldfish. More direct comparative studies will be necessary to determine the order of potency of these GH-releasing factors. A brain and pituitary TRH receptor has been partially characterized in goldfish with an apparent dissociation constant of 2-4 nM (Burt and Ajah, 1984), which is in the range of our ED_{50} (0.5-5 nM) estimates for TRH-induced GH secretion. The GH response to TRH was specific in that TRH stimulated GH, but not GTH, release from pituitary fragments shown to have both a GH and GTH response to sGnRH. We present preliminary evidence that the *in vitro* GH response to TRH in the goldfish may be desensitized by previous exposure to TRH, since the GH release responses to increasing or decreasing doses of TRH were different. The *in vivo* GH response in chickens (Scanes and Harvey, 1988) and cattle (Kesner et al., 1977) becomes refractory to TRH. Together these data suggest that desensitization of the TRH response may be a common feature of the neuroendocrine control of GH secretion in vertebrates.

The TRH sensitivity of sexually mature females was higher than that of sexually regressed females, indicating that reproductive stage influences somatotroph secretory function in goldfish. Furthermore, the response to TRH was greater in sexually regressed females treated with E_2 than in controls. These data suggest that estrogen may be involved in modulating pituitary GH release responses to TRH. Ovariectomy and E_2 treatment in female rats decreases and increases, respectively, the *in vivo* GH response to TRH (Ojeda et al., 1977). Estrogen treatment in human males also enhances the GH response to TRH (Rutlin et al., 1977). In the chicken, however, E_2 reduces the pituitary GH response to TRH (Hall et al., 1984b). Whether these results indicate true species differences in estrogen action on TRH-induced GH secretion remains to be determined; however, it is clear that E_2 modulates the GH secretory response to TRH in mammals, birds and fish.

In goldfish, GnRH and its analogues are potent stimulators of GH release (Marchant et al., 1989a). In the present study we have confirmed these observations and demonstrate for the first time that both basal and sGnRH-stimulated GH release are positively regulated by E_2. Whether E_2 in goldfish affects the stimulatory actions of other neuropeptides is unknown. A GRF from carp brain has been characterized and stimulates GH release in goldfish (Vaughan et al., 1991), but it is not known if sex steroids affect GRF action in fish as they do in the rat (Shulman et al., 1987; Houben and Denef, 1990). Neuropeptide Y is a potent stimulator of GH secretion in goldfish and GH responsiveness is lower in sexually regressed than in sexually mature females (Peng et al., 1990). Preliminary evidence (Peng, Trudeau, Peter; unpublished data) suggests that E_2 also enhances the *in vitro* GH reponse to NPY. Interestingly, when *in vitro* GH responses to sGnRH and TRH were expressed as percentage above basal to correct for E_2 induced elevations in basal secretion, the potentiating effect on secretagogue responses was not apparent. This discrepancy in data expression suggests that the total amount of GH secretion in response to sGnRH and TRH is enhanced by E_2, but that the sensitivity to neuropeptide remains unaltered. Future studies will address the site and mechanism of estrogenic modulation of neuropeptide-stimulated GH secretion in goldfish.

An increased basal output of GH *in vivo*could also be indicative of a reduced sensitivity to inhibitory factors. Somatostatin is a potent inhibitor of GH secretion in the goldfish (Marchant et al., 1987) and the positive effect of E_2 on GH may be indicative of changes in the action or quantity of this neuropeptide. In rats, SRIF inhibits *in vitro* GH secretion from dispersed cells of T treated animals but not from cells of E_2 treated animals (Hertz et al., 1989) and gonadal steroids can affect GH secretion by alteration of hypothalamic SRIF production (Argente et al.,1990; Werner et al., 1988). Cook and Peter (1984) reported that the inhibitory action of SRIF *in vivo* was more pronounced in sexually mature male goldfish than in sexually regressed female goldfish, thus providing circumstantial evidence of sex steroid modulation of SRIF action in this species. Furthermore, as female goldfish progress through gonadal recrudescence and reach gonadal maturity in spring, there is an increase in serum GH and a decrease in hypothalamic and telencephalon, and pituitary SRIF content (Marchant et al., 1989b), suggesting a relationship between gonadal function, GH secretion and SRIF production.

A novel aspect of the present study is the clear differential effect of T and E_2 on *in vivo* GH secretion in the goldfish. In rats, T and E_2 have been shown to have opposite effects on GH secretion (Hertz et al., 1989; Jansson et al., 1985; Shulman et al., 1987; Thorner et al., 1986). In our study on goldfish, T had no effect whereas E_2 stimulated GH secretion throughout the year. These observations are difficult to explain since aromatase activity in the brain and pituitary ensures efficient conversion of androgen to estrogen in neural tissues of the goldfish (Callard, 1983). The lack of T effect on GH secretion is not a result of inadequate T release from pellets or a failure of T to be aromatized, since T implantation elevates serum T levels and T via aromatization to E_2, potentiates the serum GTH response to GnRH in goldfish (Trudeau et al., 1991). It may be that T is aromatized within neural tissues not associated with control of GH secretion or that peripheral (circulating) versus neural (local aromatization of T) sources of E_2 differentially affect GH control sites. Given the differential effects of E_2 and T, the goldfish may represent a unique vertebrate model in which to study the actions of sex steroids in the control of GH secretion.

In summary, gonadal recrudescence in the female goldfish during the fall and winter is accompanied by increased serum levels of GTH (Habibi et al., 1989; Trudeau et al., 1991), GH (Marchant and Peter, 1986 and this study) and sex steroids (Kobayashi et al., 1986). The present results clearly demonstrate the positive action of E_2 on basal, sGnRH- and TRH-stimulated GH secretion. Recently, GH has been shown to potentiate the steroidogenic action of GTH by increasing the production of both T and E_2 in goldfish ovarian follicles (Van Der Kraak et al., 1990). Furthermore, E_2 enhances GnRH-induced GTH secretion in goldfish (Trudeau et al., 1991). Taken together, these results indicate the existence of a physiological relationship between GH, GTH and E_2 secretion in female goldfish.

Table 6.1. The effects of 5 day implantation of estradiol (100 µg/g) and testosterone (100 µg/g) on serum GH (ng/ml) in female goldfish. Data are presented as mean ± SE (n=5-12 per group)

Treatment	Blank	Estradiol	Testosterone
Date			
Apr 1988	96.5 ± 19	281.6 ± 73.8*	NP
May	165.7 ± 24.7	784.7 ± 154.4*	151.5 ± 48.5
July	48.4 ± 10.4	182.4 ± 63.5*	NP
Oct	84.4 ± 33.3	389.5 ± 106.5*	82.4 ± 11.3
Feb 1989	127.9 ± 21.8	420.5 ± 84.2*	138.3 ± 30.9
March	95.2 ± 15.7	230.2 ± 35.3*	141.3 ± 19.9
May	140.0 ± 41.5	545.6 ± 100.6*	201.4 ± 45.3
Oct	48.8 ± 10.6	241.6 ± 61.3*	80.0 ± 26.3

*- Estradiol elevated serum GH above values for Blank implanted fish ($p<0.05$)

NP-experiment not performed

Table 6.2. The effects of 5 day implantation of increasing doses of estradiol (25, 50, 75 and 100 μg/g) on serum GH concentration (ng/ml) in post-spawning (June) female goldfish. Data are presented as mean ± SE (n=8-11 per group).

Treatment	Serum GH (ng/ml)
Blank	62.3 ± 8.5^{c}
E2 25	130.3 ± 21.8^{b}
E2 50	110.4 ± 21.7^{b}
E2 75	137.8 ± 23.5^{b}
E2 100	212.8 ± 36.6^{a}

a,b,c- means with different superscripts are significantly different ($p<0.05$).

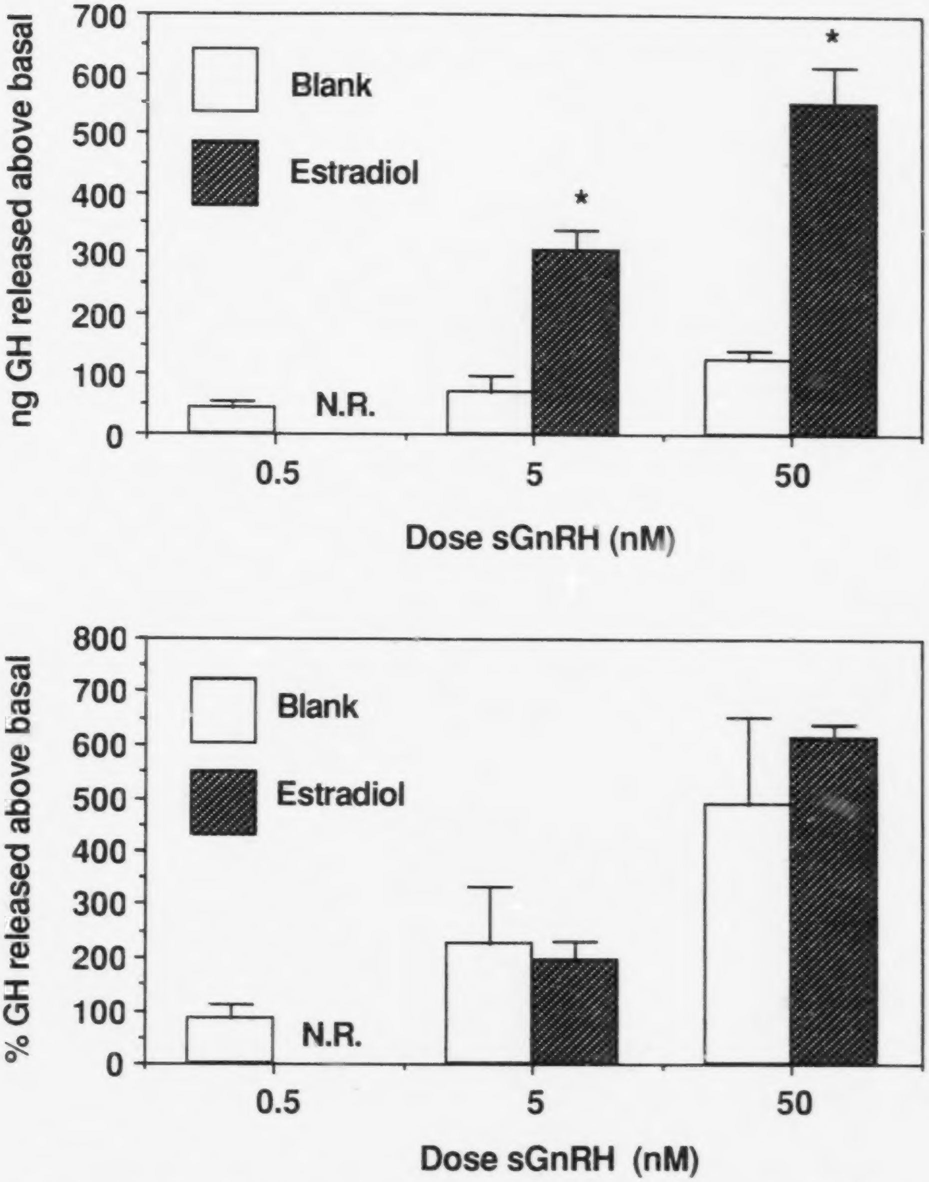

Fig. 6.1. The effect of intraperitoneal implantation of E_2 (100 μg/g) on the *in vitro* GH release response to 2-minute pulses of sGnRH. Pars distalis fragments from sexually regressed female goldfish were used. Data are the mean ± SE (n=3 columns) of the total ng GH released above basal (top panel) or the % GH released above basal (bottom panel). The (*) indicates a significant ($p<0.05$) effect of E_2 on sGnRH-induced GH secretion. N.R.= no response above basal.

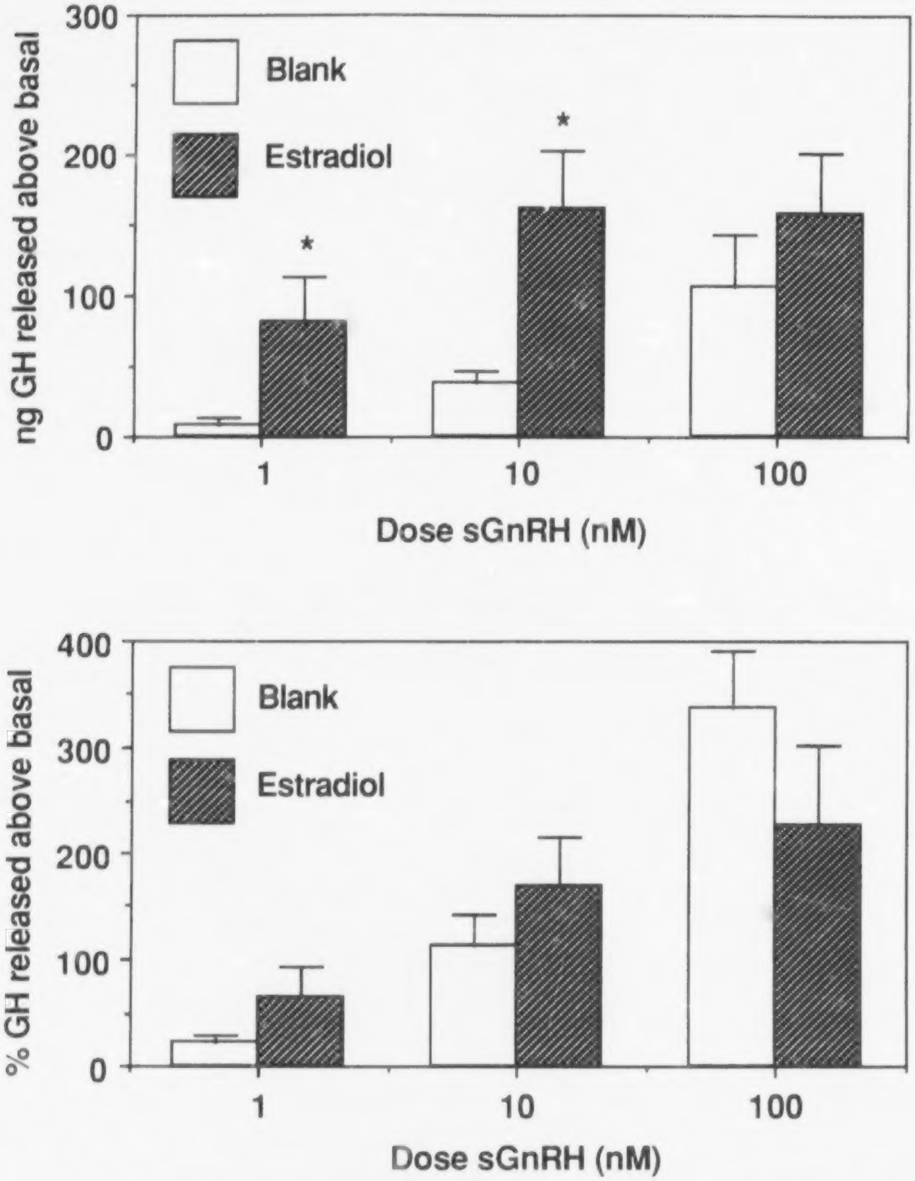

Fig. 6.2. The effect of intraperitoneal implantation of E_2 (100 µg/g) on the *in vitro* GH release response to 2-minute pulses sGnRH. Pars distalis fragments from sexually recrudescent female goldfish were used. Data are the mean ± SE (n=4-5 columns) of the total ng GH released above basal (top panel) or the % GH released above basal (bottom panel). The (*) indicates a significant ($p<0.05$) effect of E_2 on sGnRH induced GH secretion.

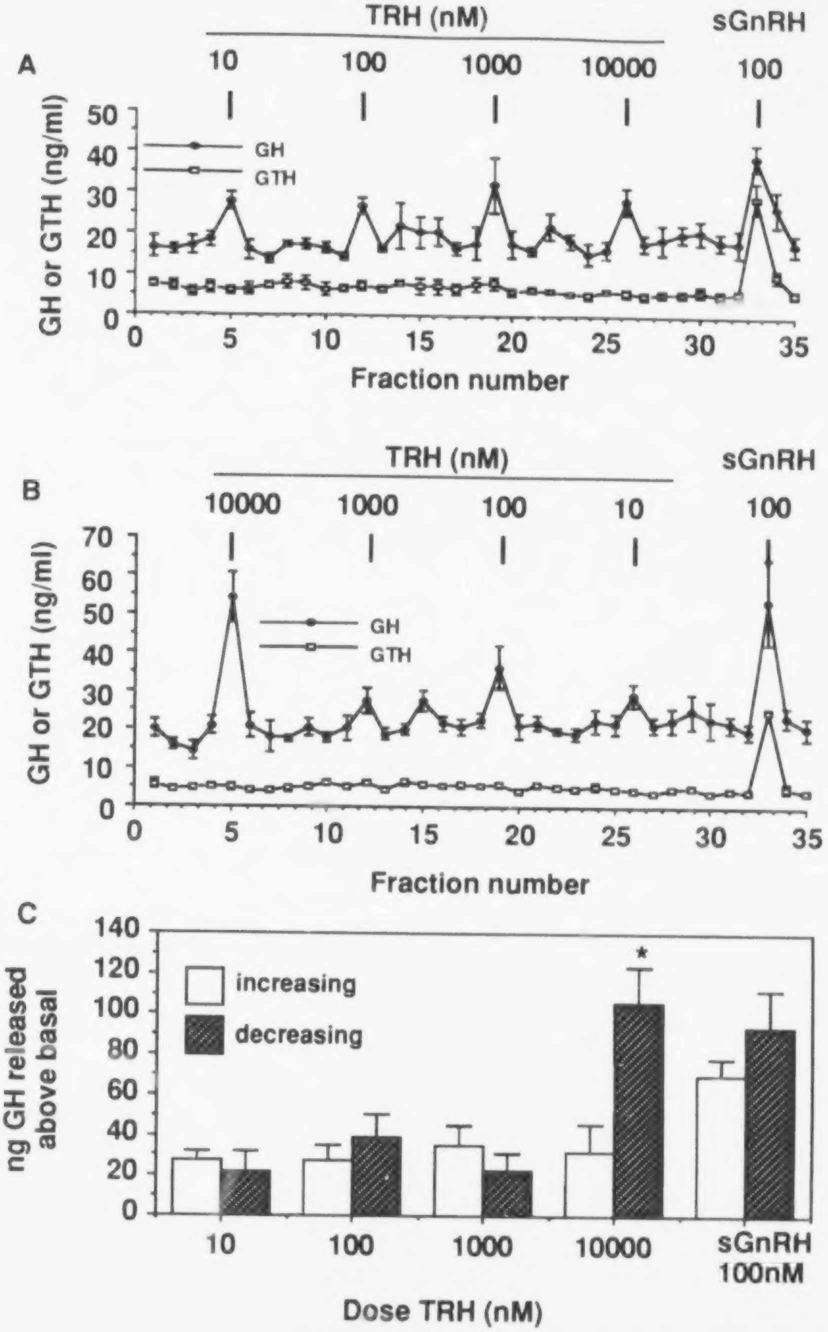

Fig. 6.3. The effects of 10 minute pulses of TRH administered in increasing (panel A) or decreasing (panel B) order on GH and GTH secretion from pars distalis fragments of sexually mature female goldfish. A 2 minute pulse of 100 nM sGnRH was given at the end. Total ng GH released above basal are depicted in panel C. The (*) indicates a significant ($p<0.05$) difference in the amount of GH released in response to 10000 nM TRH.

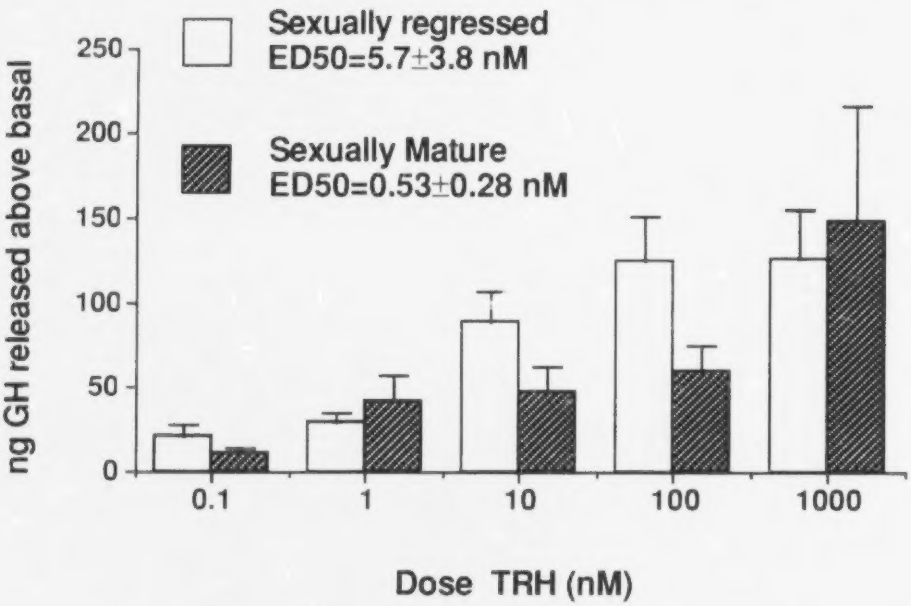

Fig. 6.4. The effect of 2 minute pulses of TRH on GH secretion from pars distalis fragments of sexually regressed or sexually mature female goldfish.

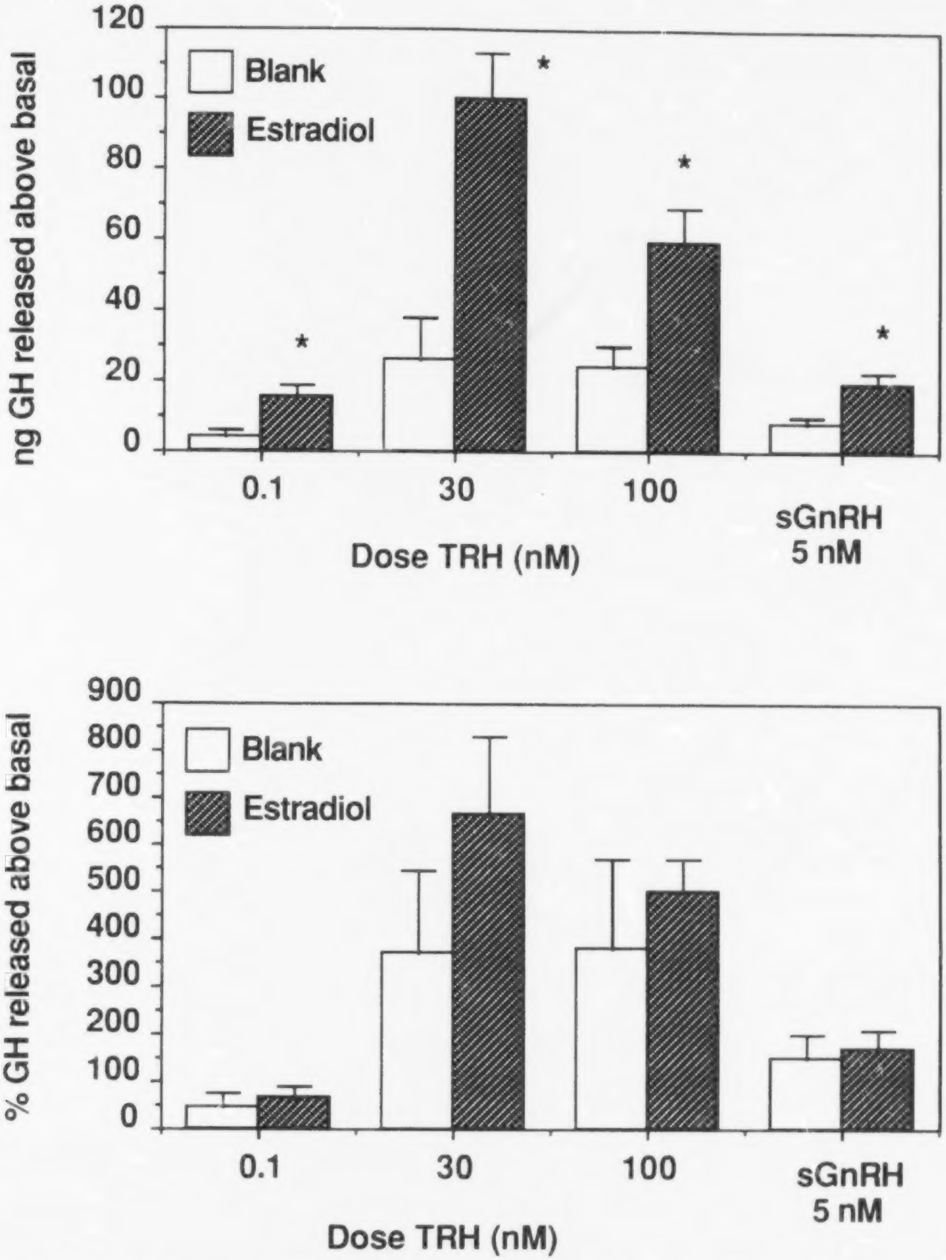

Fig. 6.5. The effect of intraperitoneal implantation of E_2 (100 µg/g) on the *in vitro* GH release response to 2-minute pulses of TRH and sGnRH. Pars distalis fragments from sexually regressed female goldfish were used. Data are the mean ± SE (n=4-5 columns) of the total ng GH released above basal (top panel) or the % GH released above basal (bottom panel). The (*) indicates a significant ($p<0.05$) effect of E_2 on TRH and sGnRH-induced GH secretion.

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7. GENERAL DISCUSSION

Early literature on the effects of sex steroids in teleosts was based primarily on observations of either positive or negative covariations in circulating levels of gonadotropin and sex steroids (reviewed in Chapter 1). These data provide essential information on the life histories of a variety of teleost species, but do not examine sex steroid actions on GTH secretion directly. Gonadectomy in salmonids (Bommelaer et al., 1981) and goldfish (Kobayashi and Stacey, 1990) generally results in increases in serum GTH levels *in vivo*, but responses are variable. The question posed in Chapter 2 is whether sex steroids affect GTH release in goldfish that have not been surgically altered. Initial observations (data not shown) were disappointing since implantation of testosterone (T) or estradiol (E_2) for up to 2 weeks did not affect unstimulated serum GTH levels. However, as outlined in Chapters 2 and 3, T via aromatization to estrogen has profound effects on pituitary responsiveness to gonadotropin-releasing hormone (GnRH) without affecting basal GTH levels. Interestingly, E_2 positive action in vivo was noted only in sexually regressed fish or in fish in early stages of gonadal recrudescence. In contrast T positive action was observed throughout the entire seasonal reproductive cycle of the goldfish. The reason for this differential response was not addressed in the present studies but could relate to differential uptake of T versus E_2 into brain and pituitary tissues (Pardridge, 1981; Callard and Gelinas, 1991). In addition, preliminary studies have also implicated progesterone (P_4) in the feedback control of GTH in goldfish (Appendix 2). Progesterone was found to enhance pituitary responsiveness to GnRH by potentiating the E_2 positive action. These potentiating effects of sex steroids may be part of a positive feedback loop in the gonad-intact goldfish, since mimicking elevations in GTH with chronic injections of human chorionic gonadotropin (hCG) also enhances GTH responses to GnRH. I have extended my observations on the positive action of T on GTH secretion in goldfish to the common carp and Chinese loach (Appendix 1). The potentiating effect of T on GnRH-induced GTH secretion may be a common feature of neuroendocrine control of GTH release in adult teleosts.

The *in vivo* steroid implantation protocol described in Chapter 2 elevates serum T and E_2 levels to values similar to those at ovulation in goldfish (Kobayashi et al., 1987). The positive effects of *in vivo* steroid treatments are maintained *in vitro* in the absence of supplemental T and E_2. The potentiating effects of sex steroids on GTH release are observed for both salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II), the endogenous GnRH peptides in the goldfish brain and pituitary (Yu et al., 1988). The positive effects of T on sGnRH-induced GTH secretion *in vitro* can be blocked by cycloheximide (Chapter 3), demonstrating that protein synthesis is involved. The specific mechanisms involved in the positive effects of T are not clear; however, the effects of T appear to be independent of changes in pituitary content of immunoreactive GTH, GnRH receptor affinity or binding capacity. It has been shown in the rat (Liu and Jackson, 1988; 1990) and chicken (King et al., 1989) that sex steroids can have profound effects on the intracellular cascade of molecular events leading to secretion of GTH. In addition, the intracellular signal transduction pathways involved in sGnRH and cGnRH-II stimulated GTH release from goldfish piuitary cells are different (Chang et al., 1991). It would be of interest to determine if these post-receptor mechanisms in gonadotrophs are enhanced by sex steroids, thereby leading to greater responsiveness to sGnRH and cGnRH-II.

Sex steroids have been shown to affect GnRH levels in the hypothalamus of mammals (Kalra and Kalra, 1989) and immature teleosts (Dufour et al.,1985; Goos et al., 1986) and their actions on the GnRH system could affect GTH production and release. There is an inverse relationship between gonadal size and serum GTH levels, and brain GnRH content, since total hypothalamic and pituitary GnRH contents (sGnRH plus cGnRH-II) decrease during gonadal recrudescence in female goldfish (Yu et al., 1987). In contrast, implantation of T and E_2 in gonad-intact goldfish did not significantly alter brain and pituitary sGnRH levels or potassium-stimulated sGnRH release from brain and pituitary slices *in vitro* (Chapter 3), nor did sex steroid treatments affect basal GTH levels in goldfish serum (Chapter 2). These observations suggest that enhanced pituitary responsiveness to GnRH following T and E_2 implantation for 5 days is not mediated by changes in endogenous sGnRH levels. It may be that longer treatments with sex steroids are necessary to affect brain and pituitary GnRH levels. Furthermore, sGnRH and cGnRH-II are differentially distributed in the goldfish brain and pituitary (Yu et al., 1988) and sex steroids may differentially regulate sGnRH versus cGnRH-II levels in the adult goldfish.

Dopamine (DA) is the primary inhibitory component in the neuroendocrine control of GTH secretion in the goldfish (Peter et al., 1986). It has been suggested that to catabolize DA fish use catechol-O-methyl-transferase (COMT; Nilsson, 1989; Saligaut et al., 1990) in contrast to mammals which use primarily monoamine oxidase (MAO; Yu, 1986). This led to the hypothesis that negative feedback effects of estrogens are mediated by their conversion to catecholestrogens (CE), and that CE in turn competes with DA for COMT, leading to an increase in DA action to inhibit GTH release (De Leeuw et al., 1987; Timmers et al., 1989). The implicit assumption of this work is that COMT is more important than MAO in catabolism of DA in fish brain. However, Sloley et al. (1992) have shown that MAO is the major metabolic pathway for DA degradation in goldfish brain and pituitary, and that CE do not affect DA catabolism. Furthermore, treatment of goldfish with high doses of CE do not affect GTH secretion in goldfish (Chapter 2).

Sex steroids interact with catecholaminergic systems in the goldfish by altering DA and NE turnover rates in the brain and pituitary (Chapter 4). The significance of changes in brain CA turnover are not apparent from the present studies; however, any changes in DA or NE turnover in the telencephalon and hypothalamus would be expected to affect the functioning of GnRH neurons since DA inhibits and NE stimulates GnRH release from the preoptic-anterior hypothalamus in goldfish (Yu et al., 1991b; Yu and Peter, 1991). I also found that sex steroids increase pituitary DA turnover. DA is a potent inhibitor of basal and GnRH-induced GTH release (Peter et al., 1986) and increased pituitary DA turnover may act to reduce stimulatory actions of endogenous GnRH, thus preventing excess GTH release and maintaining low basal GTH secretion in spite of enhanced pituitary responsiveness to GnRH and domperidone (and possibly other stimulatory agents). Injection of the DA antagonist domperidone (DOM) increases pituitary GnRH receptor capacity in goldfish (DeLeeuw et al., 1989). Therefore, steroid-induced increases in pituitary DA turnover rate may also function to regulate GnRH receptor capacity. Since treatments with DOM (DeLeeuw et al., 1989) or GnRH (Omeljaniuk et al., 1989) both upregulate GnRH receptor binding, it would be of interest to examine GnRH receptor changes in the DOM or GnRH-injected, steroid-primed paradigm. It may be that during a GTH surge, there is a forward feeding cascade that results in decreased DA turnover (Dulka et al., 1992) and increased GnRH release (Yu et al., 1987; Yu et al., 1991a) to upregulate GnRH receptors. Since ovariectomized or sexually regressed female goldfish implanted with T or E_2 respond to environmental cues (plants and increased water temperature) with an ovulation-like GTH surge (Kobayashi et al., 1989), sex steroids may be involved in priming the neural trigger for GTH surge release in this species.

The goldfish brain and pitutary contain the amino acid neurotransmitters γ-amino butyric acid (GABA), glutamate (GLU) and taurine (TAU), and experiments outlined in Chapter 5 (see also Kah et al., 1992 and Sloley et al., 1992) demonstrate that these substances may be important in the neuroendocrine regulation of reproduction in the goldfish. In the goldfish, intracerebroventricular (Sloley et al., 1991) and i.p. injection of GABA, and elevation of brain and pituitary GABA levels following i.p. GVG injection cause an increase in serum GTH levels. In marked contrast, GABA has both inhibitory and stimulatory effects on GTH release in rats. Whereas peripheral injection may be stimulatory, central injection of GABA is often inhibitory to luteinizing hormone (LH) release in rats (Masotto and Negro-Vilar, 1986; McCann and Rettori, 1988). In the rat, GVG treatment causes an increase in GABA levels in all brain areas but in contrast to the situation in goldfish, GVG causes a decrease in GTH secretion in rats (Donoso et al., 1986). Since DA is a potent inhibitor of GTH and GnRH release in goldfish, GABA could act to inhibit dopaminergic systems (McGeer and McGeer, 1989; Mansky et al., 1982), thereby stimulating GTH release. Inhibition of catecholamine synthesis with α–methyl tyrosine resulted in a potentiated GTH response to the GABA transaminase inhibitor, GVG, suggesting that GABA does not only act to stimulate GTH release by modulation of CA neurotransmission (Chapter 5). GABA stimulates GnRH release from goldfish pituitary fragments *in vitro* (Kah et al., 1992) and stimulates GTH release when administered into the third brain ventricle (Sloley et al., 1992). Together, these studies suggest that GABA somehow acts on the GnRH neuron to stimulate GTH release in goldfish. Future research should be directed towards understanding GABA actions on GnRH systems in the goldfish brain and pituitary.

Sex steroids modulate GABA action by altering pituitary responsiveness to GABA and by regulating GABA synthesis rates in the brain and pituitary. In particular, E_2 increases GABA synthesis in the hypothalamus and pituitary (Chapter 5), while reducing pituitary GTH release-responses to GABA (Kah et al., 1992). In contrast, T decreases GABA synthesis rate in the telencephalon-preoptic area and pituitary while enhancing pituitary GTH release-responses to GABA. P_4 also decreased GABA synthesis in the telencephalon-preoptic area and pituitary but did not affect pituitary GTH release-responses to GABA. These studies demonstrate that GABAergic neurons in the brain and pituitary of goldfish are steroid-sensitive, and sex steroid feedback action likely involves changes in both GABA synthesis and GABA-induced pituitary GTH release. GABA is also involved in the regulation of sexual behaviour in vertebrates (Sales et al., 1986) and steroid-mediated sexual activity could also involve GABAergic systems.

Taurine is a sulphur containing amino acid that is ubiquitously distributed in neural and non-neural tissues in vertebrates (Huxtable, 1989). Its role in regulation of reproduction remains virtually unexplored in any experimental model. Studies outlined in Chapter 5 demonstrate that TAU has an important stimulatory role on GTH release in goldfish and that sex steroids potentiate this action. The mechanism of action of TAU is unknown but could involve effects on the GnRH neuronal system in the goldfish brain and pituitary, analogous to that proposed for GABA.

Preliminary studies on the role of glutamate were initiated and this amino acid may also be involved in GTH release in goldfish. Glutamate may act through multiple excitatory amino acid receptor subtypes to influence LH secretion in rats (Bourguignon et al., 1989; Nemeroff et al., 1985). In my studies with goldfish, only N-methyl-D,L-asparate (NMA) receptor-mediated actions on GTH release were examined. It was found that NMA had a slight stimulatory effect on GTH release and in contrast to GABA and TAU, its actions were not modulated by sex steroids.

Gonadal recrudescence in female goldfish during the fall and winter is accompanied by increased serum levels of GTH (Habibi et al., 1989 and Chapter 2), growth hormone (GH; Marchant et al. 1986, and Chapter 6) and sex steroids (Kobayashi et al., 1986). In contrast to basal serum GTH levels, E_2 but not T increases basal serum GH levels *in vivo* (Chapter 6). This may involve increased responsiveness to putative GH releasing factors because E_2 treatment *in vivo* enhances GH reponsiveness to GnRH and TRH *in vitro*. GnRH in goldfish stimulates GTH and GH release. In contrast, TRH is a potent stimulator of GH release without actions on GTH release. It is not likely that E_2 increases serum GH by an effect on GnRH since 5 day treatments with E_2 did not affect brain or pituitary GnRH levels in goldfish (Chapter 3). Enhanced TRH production or increased pituitary

TRH receptor numbers are other possible mechanisms for E_2 action on GH secretion in goldfish as sex steroids have been shown to affect these parametes in mammalian tissues (Bhasin et al, 1984 ; Sharif, 1988). Somatostatin is a potent inhibitor of GH secretion in goldfish (Marchant et al., 1987) and the positive effect of E_2 on GH may be indicative of changes in the action or quantity of this neuropeptide (Hertz et al., 1989; Werner et al., 1988; Argente et al., 1990). Furthermore, DA has important stimulatory effects on GH release in goldfish (Chang et al., 1990). It is not likely that E_2 stimulates GH secretion by stimulatory actions on DA neurons, as both T and E_2 increase pituitary DA turnover but only E_2 stimulates GH release.

The major findings of this thesis have been incorporated into a model for the actions of sex steroids in the neuroendocrine regulation of GTH and GH release in goldfish (Fig. 7.1). At the beginning of gonadal recrudescence, increasing blood levels of gonadal steroids act to prime the pituitary, and initiate a positive feedback loop for the upregulation of GTH secretion. Further development of steroidogenic tissues in the gonad results in enhanced sex steroid production and additional enhancement of the functioning of the pituitary. Concurrently, the sex steroids modulate both inhibitory (DA) and stimulatory (NE) catecholaminergic systems, thereby further activating the neuroendocrine control on GTH secretion. Increased sex steroid secretion may also act to modulate GABA synthesis, and GABA- and TAU-dependent GTH release. In addition, during gonadal recrudescence, increasing levels of E_2 act to enhance GH secretion, which act in concert with GTH to promote gonadal function and growth (Van Der Kraak et al., 1990). At the time of spawning, a highly primed brain-pituitary axis responds to environmental and pheromonal cues to initiate surge release of GTH and GH (Yu et al., 1991) and stimulate the final stages of oocyte maturation and ovulation. The pituitary-gonadal axis regresses following spawning, but a highly primed GH secretory mechanism continues to release high levels of GH for several months. At a time when water temperatures are increasing during the early summer, somatic growth is accelerated (Marchant and Peter, 1986). In late summer GH levels decline, and in the autumn GTH and GH increase again at the onset of gonadal recrudescence when the seasonal reproductive cycle resumes.

The proposed model does not show the relative importance of positive and negative sex steroid actions, as these mechanisms function concurrently to regulate pituitary function in the gonad-intact, adult goldfish. Gonadal steroids may act to modulate the relative contributions of stimulatory an/or inhibitory neuroendocrine signals which ultimately determine the patterns of GTH and GH secretion. The model is intended to serve as a guideline for future research on the interactions of sex steroids and neuroendocrine factors regulating reproduction and growth in teleosts.

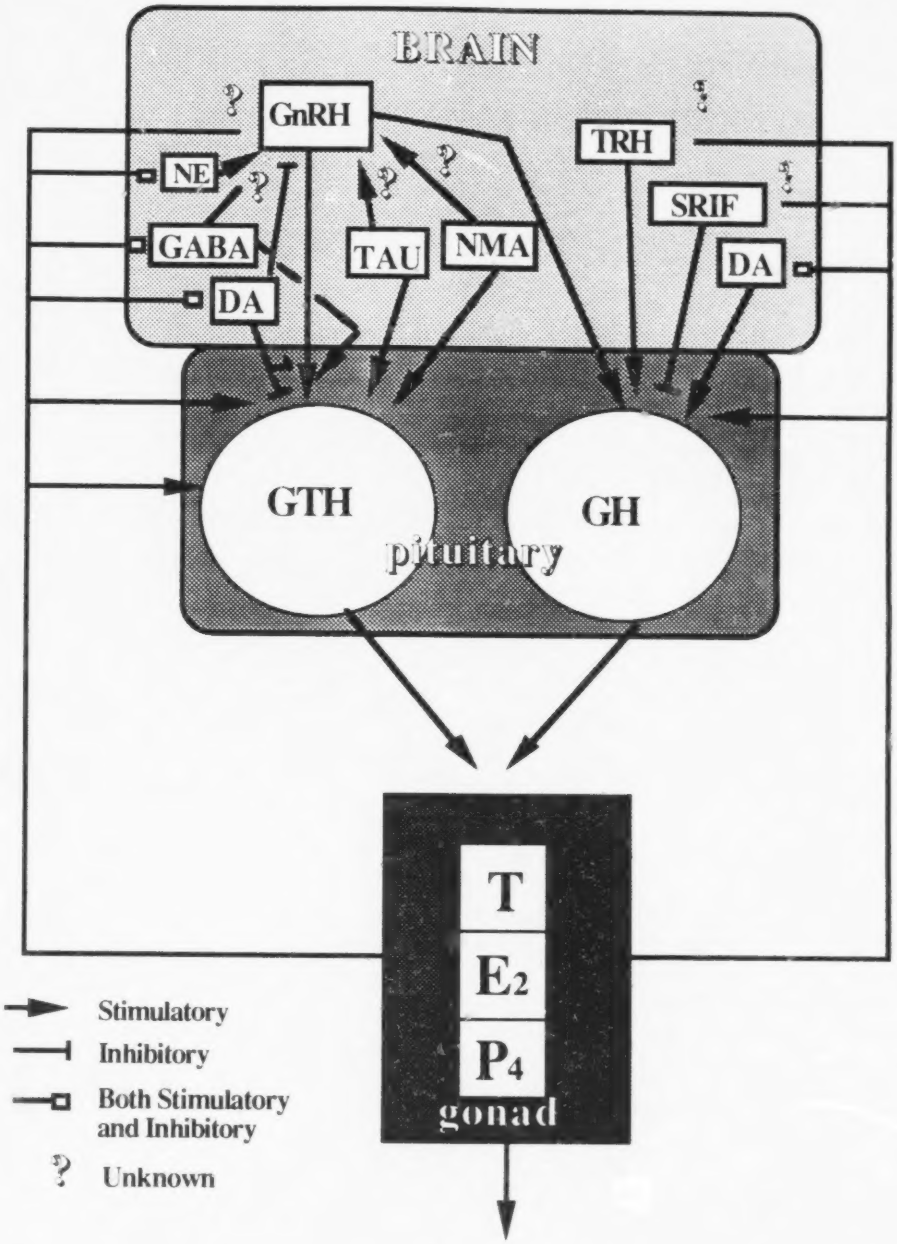
BRAIN
GnRH
NE
GABA
DA
TAU
NMA
TRH
SRIF
DA
?
GTH
GH
pituitary
T
E2
P4
gonad
Gametes
Stimulatory
Inhibitory
Both Stimulatory and Inhibitory
Unknown

Fig. 7.1. Proposed model for the actions of sex steroids in the neuroendocrine regulation of GTH and GH secretion in the goldfish. DA=dopamine; E_2=estradiol; GABA=γ-amino butyric acid; GnRH=gonadotropin-releasing hormone; NE=norepinephrine; NMA= N-methyl-D,L-aspartate; P_4=progesterone; T= testosterone; TAU=taurine; TRH=thyrotropin-releasing hormone.

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8. APPENDIX 1

TESTOSTERONE POTENTIATES THE SERUM GONADOTROPIN RESPONSE TO GONADOTROPIN-RELEASING HORMONE IN THE COMMON CARP *(Cyprinus carpio)* AND CHINESE LOACH *(Paramisgurnus dabryanus)*

(A version of this chapter has been accepted for publication. Trudeau, Lin and Peter, 1991. Can. J. Zool. 69: (in press)

Abstract

The effects of gonadal steroids on gonadosomatic index (GSI; gonad wt/ total body wt X 100), pituitary gonadotropin (GTH) content and serum GTH response to ([D-Ala^6, Pro^9, N-ethylamide]-luteinizing hormone releasing hormone (LHRH-A) were investigated in common carp and Chinese loach. Gonad-intact female fish were implanted intraperitoneally (i.p) for 5 days with silastic pellets containing no steroid (blank), testosterone (T; 100 μg/g) or estradiol (E_2; 100 μg/g). The serum gonadotropin (GTH) response at 6 h following i.p. injection of saline or 0.1 μg/g LHRH-A was assessed. In blank implanted female common carp i.p. injection of LHRH-A increased serum GTH levels approximately 4-, 13- and 2- fold above saline injected controls in sexually recrudescent, preovulatory and post-spawning females, respectively. Implantation of E_2 in female carp did not affect basal or LHRH-A induced GTH secretion at any time of the year. Implantation of T did not affect basal GTH levels but potentiated the GTH response to LHRH-A in sexually recrudescent and preovulatory female carp but not in post-spawning female carp. Injection of LHRH-A stimulated GTH release in female Chinese loach both prior to and in the middle of the spawning period. Treatment with T or E_2 did not affect basal GTH levels. Implantation of T but not E_2 potentiated LHRH-A stimulated GTH release at both times tested. These results demonstrate that T but not E_2 can increase pituitary responsiveness to exogenous LHRH-A in sexually recrudescent and sexually mature female common carp and Chinese loach.

INTRODUCTION

Steroid negative feedback regulation of gonadotropin (GTH) secretion has been demonstrated in several teleost species by classical gonadectomy/steroid replacement models. Elevations of blood GTH levels caused by gonadectomy in trout (Bommelaer *et al.* 1981), African catfish (Habibi *et al.* 1989) and goldfish (Kobayashi and Stacey 1990) are suppressible by testosterone (T) or estradiol (E_2). Implantation of antiestrogens into the brain and pituitary of intact goldfish stimulated GTH release (Billard and Peter 1977), which has been interpreted as further evidence for steroid negative feedback; pituitary implantation, however, may have damaged the pituitary stalk and disrupted inhibitory dopaminergic inputs into the pituitary (Peter *et al.* 1986) and caused GTH release.

Evidence for the positive effects of gonadal steroids on GTH secretion in teleosts comes primarily from studies of immature salmonids and immature eels. Increased pituitary GTH content occured following intraperitoneal administration of T in juvenile male and female rainbow trout (Crim and Evans 1979). The positive feedback effect of T is dependent on aromatization since this response was blocked by an aromatase inhibitor (Crim *et al.* 1981). Athough no changes in plasma GTH were noted in initial studies, implantation of juvenile rainbow trout with T-containing silastic capsules resulted in increased plasma GTH after 2 months (Crim and Evans 1983). Positive effects of E_2 on development of GTH cells has also been demonstrated in the pituitary of immature European eel (Olivereau and Olivereau 1979; Dufour *et al.* 1983) and Japanese eel (Lin *et al.* 1990).

The positive effects of gonadal steroids on GTH secretion in adult fish have only been studied in gonad-intact goldfish. In the both male and female goldfish, both T and E_2 exert a positive effect on GnRH-induced but not basal GTH levels (Trudeau *et al.* 1991). Whereas T is effective throughout the entire seasonal reproductive cycle, E_2 positive action is confined to sexually regressed female goldfish (Trudeau et al., 1991). Whether the positive action of gonadal steroids on induced GTH secretion is limited to adult goldfish is unknown. In this paper we examine the actions of T and E_2 in two other teleost species, the common carp (Family Cyprinidae: Cyprinus carpio) and the Chinese loach (Family Cobitididae: Paramisgurnus dabryanus).

MATERIALS AND METHODS

Animals

Common carp (0.5-1.0 kg) were raised at Zhongshan University, exposed to ambient water temperature and natural photoperiod. Chinese loach (20-40g) were obtained from a local supplier in Guangdong Province, China. They were held indoors in 250-litre aquaria, exposed to ambient temperature and held under a natural photoperiod regime. Fish were fed commercial pellets once daily.

Steroid Treatment

Solid silastic pellets containing T, E_2 or no steroid (Blank) were manufactured as previously described (Pankhurst *et al.* 1986) and elastomere contained 100 mg/g of steroid. In our previous studies in goldfish (Trudeau *et al.* 1991), a steroid dose of 100 µg/g body weight was found to raise T and E_2 to levels similar to those in ovulatory female goldfish . Under tricane methanesuphonate anaesthesia, pellets were implanted intraperitoneally (i.p.) through a 2-3 mm incision in the body wall. Testosterone and E_2 were purchased from Sigma Chemical Co. (St.Louis, MO., U.S.A.).

Experimental Protocol: Effect of T or E_2 implantation on basal and LHRH-A stimulated GTH secretion

At various times of the year, at 5 days following implantation fish were injected i.p. with either saline (0.6 % NaCl) or [D-Ala^6-Pro^9-N-ethylamide]-GnRH (LHRH-A; 0.1 µg/g) for assessement of the in vivo GTH release-response. Blood samples were taken from anaesthetized fish by caudal puncture using 25 G needles 6 hours after injection. The time course of the GTH response to LHRH-A is well established in goldfish (Peter *et al.* 1986) and is comparable to that in common carp and Chinese loach (Lin *et al.* 1988). Blood was allowed to clot 4-6 h (at 4° C), and serum was collected by centrifugation and kept frozen (-20° C) until hormone analysis.

At the termination of an experiment, fish were sacrificed and total body and gonad weights recorded. A gonadosomatic index (GSI) was calculated as gonad weight/ total body weight X 100. Pituitaries were removed, homogenized in 1 ml radioimmunoassay buffer and stored frozen until hormonal content determination.

Radioimmunoassays

Serum GTH concentrations in common carp were determined by double antibody radioimmunoassay (RIA) as previously described (Peter *et al.* 1984). Rabbit anti-carp

GTH-II antiserum was used at a final dilution of 1:220,000. Serum and pituitary GTH concentrations in Chinese loach were determined by double antibody RIA as previously described (Lin *et al.* 1986). This assay employes a carp GTH β–subunit primary antibody at a final dilution of 1: 1,000,000. All samples (50 µl) were assayed in duplicate and, within and between assay coefficients of variation were <10%.

Statistical Analyses

Data were analysed using the least squares method of analysis of variance (AOV; Statistical Analysis Systems 1979). Serum GTH values were not normally distributed and were log-transformed prior to 2-way AOV. Post-hoc means comparisons were made between treatment groups using the least-squares means. Data for GSI and pituitary GTH contents were compared by unpaired Student's t-test. Differences between means was considered statistically significant if $p<0.05$.

RESULTS

Gonadosomatic index was determined for steroid-implanted saline-injected female carp and Chinese loach. Treatment with either T or E_2 was without effect on GSI in both species irrespective of the time of year so GSI data were pooled across treatment groups. In sexually recrudescent female carp in October the GSI was 3.0 ± 0.7 % (n=16). In preovulatory female carp in January the GSI was 20.4 ± 0.7 % (n=26). In post-spawning female carp in June the GSI was 8.8 ± 0.8 % (n=18). In late recrudescent female Chinese loach in September the GSI was 8.3 ± 0.6 % (n=17). In the middle of the spawning period in January, the GSI of sexually mature female loach was 9.2 ± 1.1 % (n=33).

The effects of steroid treatment on pituitary GTH content (µg/mg pituitary wet weight) was studied in preovulatory common carp and spawning Chinese loach in January. Pituitary GTH contents for blank, T and E_2 treated carp were not different ($p>0.05$) and were 6.0 ± 0.4 µg/ mg (n=5), 6.4 ±1.5 µg/mg (n=5) and 5.8 ± 0.5 µg/mg (n=4). Pituitary GTH contents for blank, T and E_2 treated loach were not different ($p>0.05$) and were 7.8 ± 2.3 µg/ mg (n=4), 7.8 ± 3.2 µg/mg (n=5) and 5.5 ± 0.6 µg/mg (n=5), respectively.

The effects of steroid implantation on serum GTH in saline-injected controls and LHRH-A injected female carp are presented in Figs. 1-3. In sexually recrudescent carp in October (Fig. 1) treatment with either T or E_2 did not affect GTH levels in saline injected animals. Injection of LHRH-A in blank implanted females increased GTH levels by approximately 4-fold above those in saline-injected control females. Testosterone implantation resulted in enhanced GTH release in response to LHRH-A. Estradiol implantation was without effect. In preovulatory female carp in January (Fig. 2) treatment with either T or E_2 did not affect GTH levels in saline-injected animals. LHRH-A injection increased GTH levels in blank-implanted females by approximately 13-fold. Treatment with T enhanced the GTH response to LHRH-A. In contrast, E_2 did not affect the GTH response to LHRH-A. Steroid implantation in post-spawning females in June (Fig. 3) did not affect GTH levels in saline injected fish. Injection of LHRH-A increased GTH levels in blank-implanted animals; this represented approximately a 2-fold increase in serum GTH. Both T and E_2 did not affect the GTH response to LHRH-A in post-spawning females.

The effects of steroid implantation on serum GTH in saline-injected and LHRH-A injected female Chinese loach are presented in Figs. 4 and 5. In September (Fig. 4) treatment with T or E_2 did not affect GTH levels in saline-injected females. Injection of LHRH-A in blank-implanted animals increased GTH levels. Implantation of T but not E_2 enhanced the GTH response to LHRH-A. Later in the spawning season in January (Fig. 5), a similar situation was noted. Treatment with T or E_2 did not affect GTH levels in saline-injected mature females. Injection of LHRH-A in blank-implanted animals increased GTH levels; implantation of T but not E_2, enhanced the GTH response to LHRH-A in females in the middle of the spawning period.

DISCUSSION

The results of the present study demonstrate that LHRH-A stimulates GTH secretion in female common carp and Chinese loach, confirming previous reports in teleosts (Lin *et al.* 1988; Sokolowska *et al.* 1985). In the case of common carp where experiments were performed at various times throughout the reproductive cycle, there appears to be a seasonal variation in LHRH-A induced GTH secretion. The increase in serum GTH in LHRH-A-injected blank-implanted females compared to saline-injected controls was approximately 4-fold, 13-fold and 2-fold in sexually recrudescent, preovulatory and post-spawning female carp, respectively. Seasonal variations in GnRH-induced GTH secretion have been reported for common carp (Weil *et al.* 1975), rainbow trout (Weil *et al.* 1978), and goldfish (Sokolowska *et al.* 1985). In general, the GTH response is highest just prior to or during the spawning season when GSI is greatest. In female goldfish, the GTH response to exogenous GnRH is highest just before spawning (Sokolowska *et al.* 1985; Habibi *et al.* 1989; Trudeau *et al.* 1990) when GSI and serum steroid levels (Kobayashi *et al.*1986) are maximal. Our limited seasonal study suggests that a similar relationship between GSI, gonadal steroids and GTH response to GnRH may also exist in female common carp, since the highest GTH response to LHRH-A was noted in preovulatory females (GSI of approximately 20 %).

Implantation of female carp with T and E_2 did not affect basal plasma GTH levels at any time of the year. Testosterone but not E_2 treatment resulted in enhanced GTH secretion in response to LHRH-A. The effect of T was evident in sexually recrudescent and preovulatory females, but not animals in a post-spawning condition. The reason for the lack of effect in post-spawning female carp is unknown since in post-spawning female goldfish both steroids can enhance LHRH-A induced GTH secretion. The response to E_2 in female goldfish during the post-spawning period, however, is highly variable (Trudeau and Peter, personal observations). Since ovarian condition and hence serum steroid levels are highly variable in post-spawning fish, it may be that steroid implantation did not elevate circulating T or E_2 levels consistently or sufficiently to affect pituitary responsiveness.

Implantation of female Chinese loach with T and E_2 did not affect basal plasma GTH levels. Treatment with T but not E_2 resulted in enhanced GTH secretion in response to LHRH-A. This was evident both prior to and in the middle of the spawning period. Very little is known about the reproductive cycle of the Chinese loach. The spawning season is not well defined but extends from October to April in laboratory-reared animals in Southern China. Individual females may also spawn more than once during this period (Lin, H.R., personal observations). Under natural conditions, females spawn in spring and early summer. Seasonal variations in GSI, gonadal steroid secretion and pituitary responsiveness to GnRH remains to be determined, but are likely to exist in the Chinese loach as in other teleosts. Our results suggest that increased serum T may contribute to control of reproductive function by enhancing GTH secretion in this species.

The mechanism of positive action of T on LHRH-A induced GTH secretion in adult teleosts is unknown. In immature trout (Crim and Evans 1979; 1980; 1983; Crim *et al.* 1981) and Japanese eel (Lin *et al.* 1990) T treatment increases pituitary GTH content and responiveness to GnRH, suggesting that increased pituitary content is fundamental to the increase in responsiveness. In previous work in the adult goldfish (Trudeau *et al.* 1991), steroid treatment did not affect pituitary GTH content although responsiveness to LHRH-A was increased. The lack of effect of T on pituitary GTH content in carp and loach in concurrence with an enhanced response to LHRH-A indicates that changes in pituitary GTH content are not an explanation for the present results.

It is possible that alterations in pituitary GnRH receptor numbers can account for the positive effect of T on induced GTH secretion in carp and loach, since similar steroid treatment in mammals (Clayton *et al.* 1985; Conn *et al.* 1987) and African catfish (Habibi *et al.* 1989) affects GnRH receptor number. There also exists a good positive correlation between seasonal variations in GnRH receptor binding, pituitary GnRH responsiveness, GSI and serum gonadal steroid levels in goldfish (see Habibi *et al.* 1989 and Trudeau *et al.* 1991 for discussion). We suggest that the positive action of gonadal steroids on GnRH-induced GTH secretion in adult gonad-intact teleosts may be, in part, related to changes in GnRH receptor number.

In a variety of teleost species, including common carp and Chinese loach, dopamine

(DA) has an inhibitory influence on GnRH-induced GTH secretion (Lin *et al.* 1988; Peter *et al.* 1986). It is possible that gonadal steroids may also act to modulate inhibitory catecholaminergic systems within the teleost brain and/or pituitary to enhance GTH secretion. This hypothesis is presently being examined in goldfish.

The reason for the lack of effect of E_2 in altering pituitary responsiveness to LHRH-A is unclear since T aromatization to estrogen is important for the positive action of T in other teleosts (Crim *et al*, 1981; Trudeau *et al.*, 1991). In goldfish, E_2 is only effective in enhancing pituitary responsiveness in fish that are sexually regressed or in the early stages of gonadal recrudescence (Trudeau *et al.*, 1991). Since our limited studies in carp and loach did not include sexually regressed fish, an estrogenic component to positive steroid feedback in these species cannot be excluded. Our current hypothesis is that peripheral administration of T is more effective than E_2 because T is converted locally to E_2 via an extremely active aromatase system (Pasmanik and Callard 1988) within the teleost brain and pituitary. The intra-tissue levels of estrogen after T treatment are assumed to be much higher than those produced by E_2 implantation. Clearly, future studies should be directed towards understanding seasonal variations in the positive action of T and E_2 in adult teleosts.

In conclusion, we have extended our observations on the positive action of gonadal steroids on GTH secretion in the goldfish (Trudeau *et al.* 1991) to another member of the family Cyprinidae, the common carp, and to a member of the family Cobitididae, the Chinese loach. The potentiating effect of T on GnRH-induced GTH secretion may be a common feature in adult teleost species.

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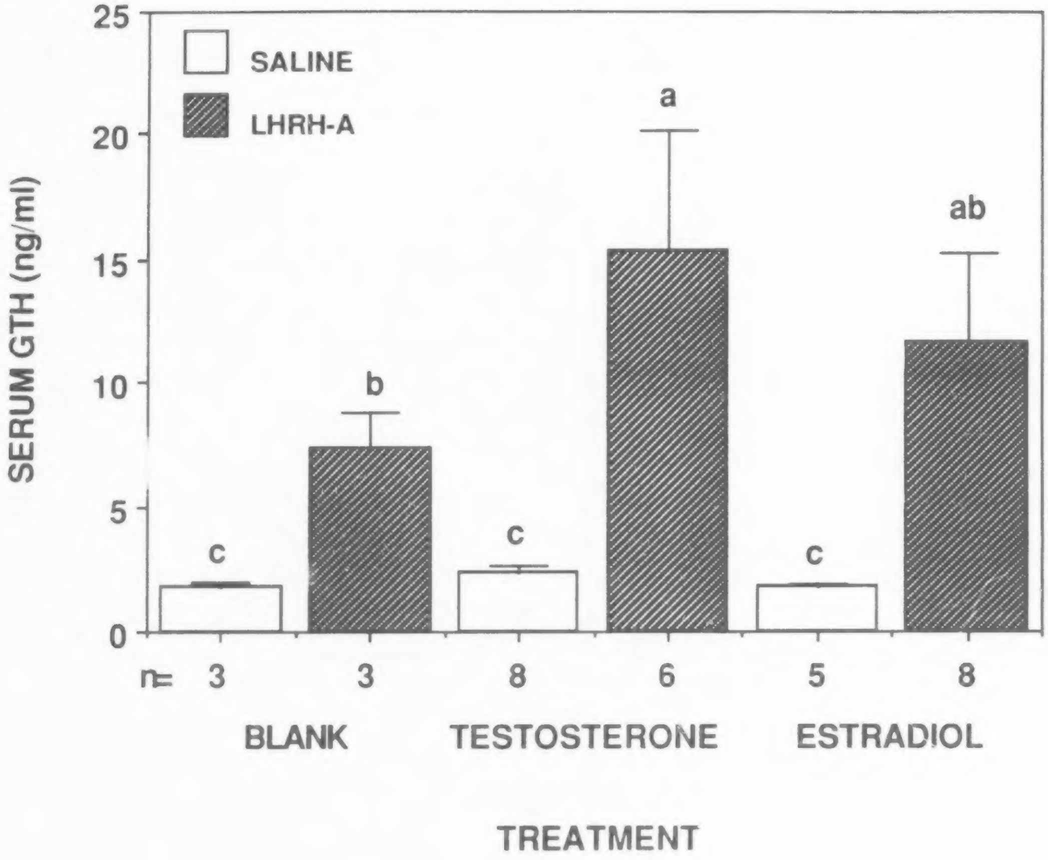

Fig. 1. The effects of testosterone and estradiol implantation on mean (±SE) serum GTH levels (ng/ml) in saline-injected (control) and LHRH-A (0.1 μg/g) injected sexually recrudescent female common carp in October. Ambient water temperature was 26-28°C. Means with different superscripts are significantlydifferent ($p<0.05$). The number of fish per group is indicated along the x-axis.

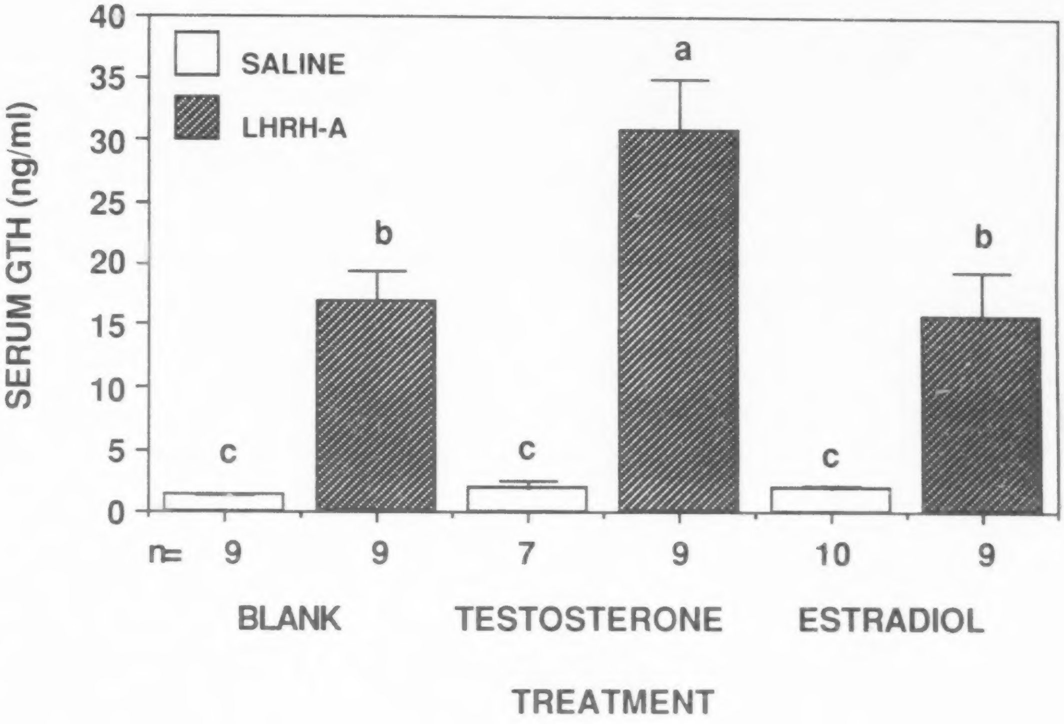

Fig. 2. The effects of testosterone and estradiol implantation on mean (±SE) serum GTH levels (ng/ml) in saline-injected (control) and LHRH-A (0.1 µg/g) injected preovulatory female common carp in January. Ambient water temperature was 13-18°C. Means with different superscripts are significantly different ($p<0.05$). The number of fish per group is indicated along the x-axis.

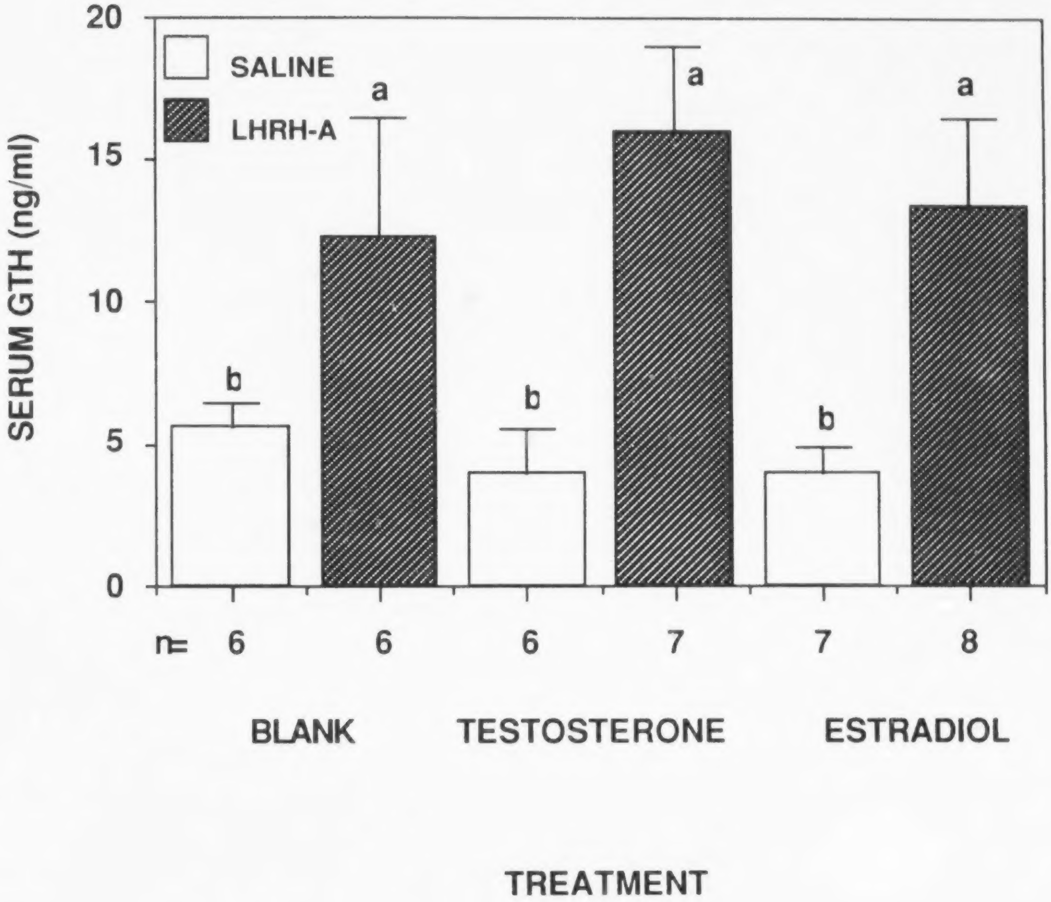

Fig. 3. The effects of testosterone and estradiol implantation on mean (±SE) serum GTH levels (ng/ml) in saline-injected (control) and LHRH-A (0.1 μg/g) injected post-spawning female common carp in June. Ambient water temperature was 25-28^{o}C. Means with different superscripts are significantly different ($p<0.05$). The number of fish per group is indicated along the x-axis.

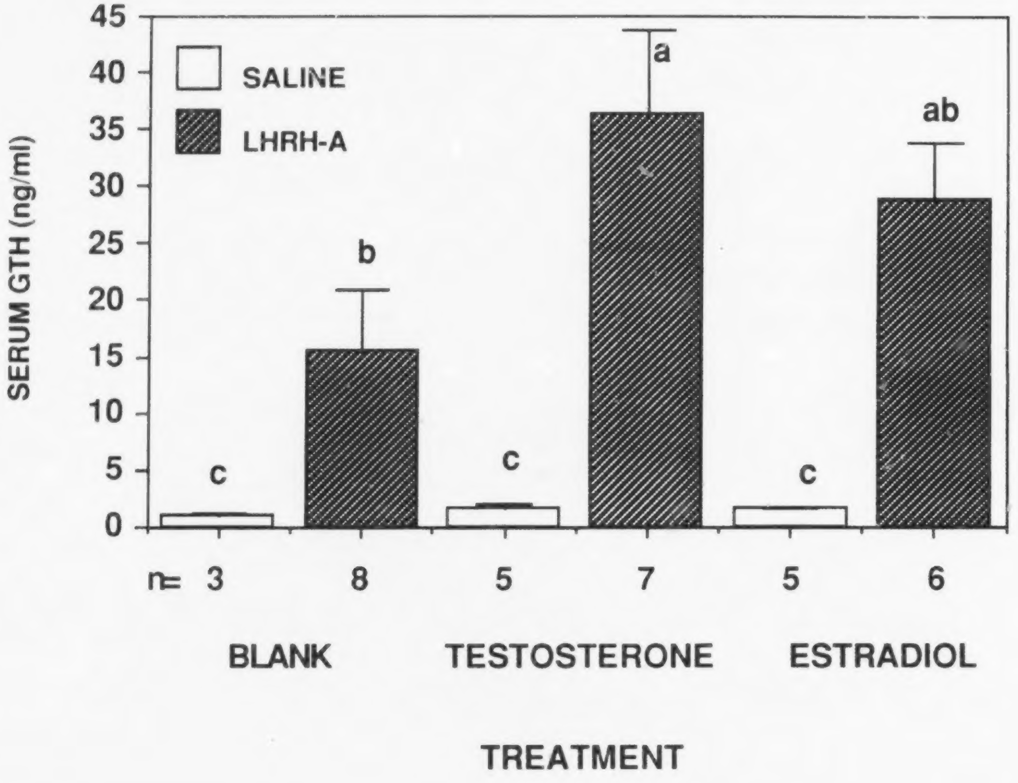

Fig. 4. The effects of testosterone and estradiol implantation on mean (±SE) serum GTH levels (ng/ml) in saline-injected (control) and LHRH-A (0.1 μg/g) injected late recrudescent female Chinese loach in September. Ambient water temperature was 27-29°C. Means with different superscripts are significantly different ($p<0.05$). The number of fish per group is indicated along the x-axis.

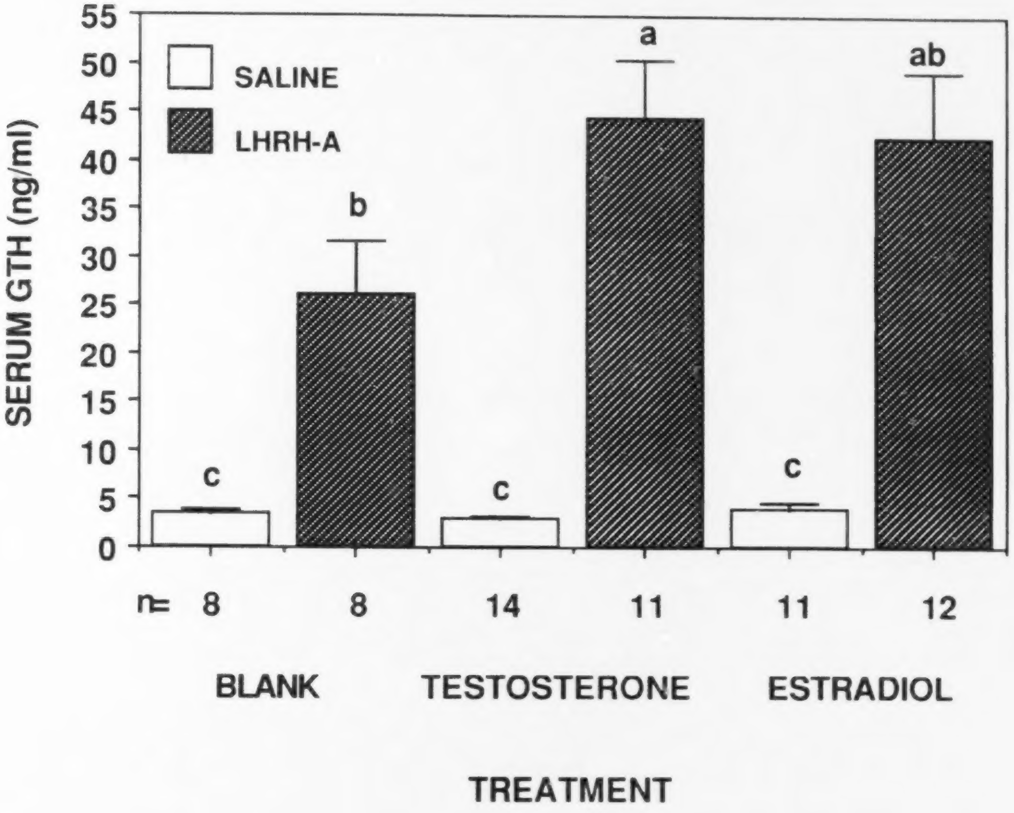

Fig. 5. The effects of testosterone and estradiol implantation on mean (±SE) serum GTH levels (ng/ml) in saline-injected (control) and LHRH-A (0.1 μg/g) injected mature female Chinese loach in the middle of the spawning season in January. Ambient water temperature was 14-17^{o}C. Means with different superscripts are significantly different ($p<0.05$). The number of fish per group is indicated along the x-axis.

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9. APPENDIX 2

MECHANISMS OF SEX STEROID NEGATIVE AND POSITIVE FEEDBACK CONTROL OF GONADOTROPIN (GTH) SECRETION IN TELEOSTS

A version of this chapter has been accepted for publication. Invited lecture: Fourth International Symposium on Reproductive Physiology of Fish. Norwich U.K. July 7-12, 1991. (Scott, A.P., Sumpter, J.P., Kime, D.E. and Rolfe, M., eds.). FishSymp 91, Sheffield, U.K., pp 224-226.

MECHANISMS OF SEX STEROID NEGATIVE AND POSITIVE FEEDBACK CONTROL OF GONADOTROPIN (GTH) SECRETION IN TELEOSTS

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Summary

Sex steroids exert both negative and positive feedback effects on GTH secretion in teleosts. This paper reviews these data and presents evidence that testosterone (T), estradiol (E_2) and progesterone (P_4) potentiate gonadotropin-releasing hormone (GnRH) induced GTH secretion. In addition, sex steroids may affect GTH secretion by modulating inhibitory dopaminergic (DA) and/or stimulatory norepinephrineric (NE) systems in the brain and pituitary.

Introduction

Steroid negative feedback regulation of GTH secretion has been demonstrated in female goldfish by classical gonadectomy/steroid replacement experiments (Kobayashi & Stacey, 1990). Until recently, evidence for steroid positive feedback was restricted to the finding that T and E_2 promote accumulation but not secretion of GTH in the pituitary (PIT) of immature salmon, trout and European eel (see Peter, 1983 for review). In gonad-intact post-pubertal goldfish, however, we have demonstrated that T through aromatization to estrogen, is involved in positive feedback regulation of GTH secretion by potentiating the GTH response to GnRH (Trudeau et al., 1991b) independent of changes in pituitary GTH content. The mechanisms underlying both positive and negative feedback effects have yet to be elucidated.

In a wide range of teleost fish there is a clear dopaminergic (DA) inhibition of GTH secretion (Peter et al., 1986) and it is possible that sex steroids regulate GTH by modulating DA-inhibition. The involvement of brain catecholamines (CA) in gonadal steroid feedback control of GTH secretion in teleosts has been suggested previously (DeLeeuw et al., 1987; Manickam and Joy, 1990; Timmers and Lambert, (1989) but there is no direct evidence for such interactions. The present report discusses recent data (Trudeau et al.,1991c) on the effects of T and E_2 on CA neuronal function. We also present new data on the interaction of progesterone (P_4) and E_2 in the control of GTH secretion in female goldfish.

Materials and Methods

At various times of the year, at 5 days following implantation (Trudeau et al., 1991b) of T, E_2 and P_4 (25-100 μg/g body wt) in silastic pellets, fish were injected i.p. with either saline (0.6 % NaCl) or [D-Ala^6-Pro^9-N-ethylamide]-GnRH (LHRH-A; 0.1 μg/g) for assessement of the serum GTH release-response. Blood samples were taken from anaesthetized fish by caudal puncture using 25 G needles 6 hours after injection. Serum GTH concentrations were determined by an established radioimmunoassay (Peter et al., 1986; Trudeau et al., 1991b). Data were analysed using the least squares method of analysis of variance.

Results

The effect of E_2 or P_4 on basal and LHRH-A stimulated GTH release in sexually regressed and early recrudescent female goldfish are shown in Fig.1.

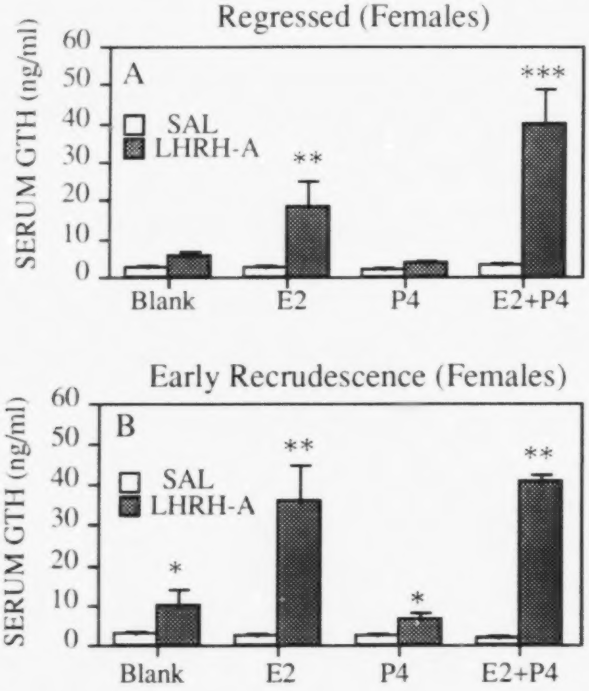

Figure 1. The effects of E_2 and P_4 (100 µg/g) on basal (saline-injected) and LHRH-A injected female goldfish in July (A) and October (B) (* LHRH-A effect, ** E_2 effect, *** P_4 potentiated the effect of E_2; $p<0.05$).

Treatment with E_2 and/or P_4 did not affect GTH levels in saline-injected animals. In sexually regressed fish, (Fig. 1, panel A) LHRH-A did not stimulate GTH release in controls but did in E_2 implanted animals. Implantation with P_4 alone did not affect the LHRH-A response but did potentiate the positive effect of E_2 on LHRH-A-simulated GTH release. In females in early stages of recrudescence, E_2 enhanced the effect of LHRH-A ; however, P_4 alone or combined with E_2 did not affect basal or LHRH-A induced GTH secretion (Fig. 1, panel B). Implantation of female goldfish in early stages of gonadal recrudescence with T does not affect basal GTH release but does potentiate LHRH-A-induced GTH secretion (Fig. 2). In another experiment with recrudescent females, E_2 did not affect basal or LHRH-A induced GTH release whereas T potentiates LHRH-A induced GTH secretion (Fig. 3). Co-implantation of E_2 with T did not affect T-positive action.

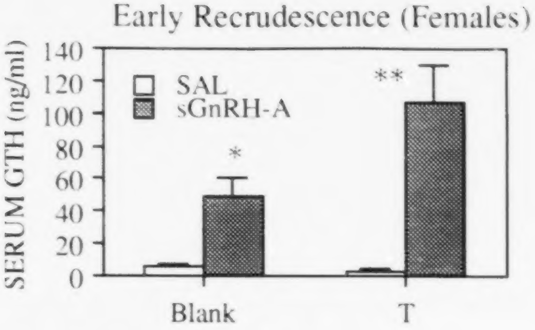

Figure 2. The effects of T (25 µg/g) on basal (saline-injected) and LHRH-A induced GTH secretion in female goldfish in November (* LHRH-A effect, ** T effect; $p<0.05$).

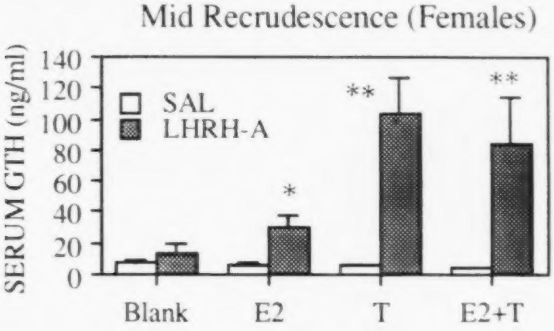

Figure 3. The effects of E_2 and T (100 µg/g) on basal and LHRH-A induced GTH secretion in female goldfish in January (*E_2 effect, ** T effect; $p<0.05$).

Discussion

In male and female goldfish with intact gonads, both T and E_2 exert a positive effect on GnRH-induced but not basal GtH secretion (Trudeau et al.,1991b; present results). Whereas T is effective throughout the entire seasonal reproductive cycle, E_2 positive action is observed only in regressed females or female goldfish in early stages of gonadal recrudescence (Trudeau et al., 1991b). In female common carp and female Chinese loach, T also potentiates the serum GTH response to GnRH (Trudeau et al., 1991a), indicating that positive feedback by sex steroids may be a common feature in control of GTH secretion in adult teleosts.

The increase in serum GTH in response to the DA antagonists pimozide (Sokolowska et al., 1985) and domperidone (Omeljaniuk et al., 1989) is greatest in sexually mature goldfish, suggesting that the DA inhibitory tone is greatest when sex steroid secretion is maximal. Recently, we have estimated DA and NE turnover rates (TOR) following CA depletion with α-methyl-p-tyrosine (Trudeau et al., 1991c) in telencephalon including preoptic area (TEL-POA) and PIT of steroid-treated female goldfish. Treatments with T and E_2 enhanced PIT DA-TOR suggesting that part of steroid negative feedback may involve increased DA inhibition of GTH release. Increased PIT DA-TOR represents a functional increase in DA inhibition since the GTH response to domperidone is concurrently enhanced by T and E_2. Since DA is a potent inhibitor of GTH (Peter et al., 1986), GnRH release (Yu et al., 1991) and GnRH receptor capacity (DeLeeuw et al., 1989), increased PIT DA-TOR may act to reduce the stimulatory action of endogenous GnRH, thus maintaining inhibitory control of GTH release in spite of enhanced PIT responsiveness to GnRH. E_2 decreased NE-TOR in TEL-POA of regressed female goldfish but increased NE-TOR in recrudescent females. In contrast, T did not affect NE-TOR in the TEL-POA of regressed females but decreased NE-TOR in the TEL-POA of recrudescent females. Implantation of T but not E_2 decreased TEL-POA DA-TOR in regressed fish. In recrudescent females, however, both steroids increased TEL-POA DA-TOR.

Steroid-induced changes in TEL-POA CA neuronal activity could act to regulate brain GnRH, since DA and NE perikarya and fibres have been localized in the preoptic area (Hornby and Piekut, 1990) in proximity to GnRH perikarya (Kah, 1986) in the goldfish. Since DA inhibits in vitro GnRH release from goldfish TEL-POA and PIT fragments (Yu and Peter, 1991; Yu et al., 1991), any alterations in TEL-POA and PIT DA-TOR could conceivably act to regulate the activity of GnRH perikarya and terminals, respectively. Furthermore, NE stimulates GnRH release from goldfish TEL-POA slices in vitro (Yu and Peter, 1991; Yu et al., 1991) and also stimulates GTH release in vivo in sexually regressed goldfish (Chang and Peter, 1984). Therefore, steroid-induced alterations in NE-TOR could also act to regulate GnRH release. In the goldfish it may be that gonadal steroids modulate GnRH and GTH release via both positive and negative actions on CA neurons.

Seasonal variations in the intensity of sex steroid negative (Bommelaer et al., 1981) and positive (Trudeau et al., 1991b) feedback is evident in teleost species. In female goldfish, the positive effect of E_2 and P_4 on GnRH-induced GTH secretion is present only in sexually regressed fish. Progesterone acts to increase pituitary responsiveness to GnRH by enhancing E_2 positive action. Serum levels of T, E_2 and P_4 increase concurrently with seasonal increases in gonadal size (Kagawa et al. 1983, Kobayashi et al. 1986,), GnRH receptor capacity (Habibi et al. 1989,) and pituitary GnRH responsiveness (Trudeau et al., 1991b) in goldfish. We suggest that during seasonal gonadal recrudescence in teleosts, sex steroids act at multiple sites within the brain-pituitary neuroendocrine axis to regulate catecholaminergic and GnRH (Yu and Peter, 1991) neuronal function, thus ensuring pituitary GTH surge release (Kobayashi et al.,1989) and successful ovulation during the spawning season.

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